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β-GLUCURONIDASE OF RABBIT POLYMORPHONUCLEAR LEUCOCYTES[1]

BY R. J. ROSSITER AND ESTHER WONG

Abstract

Rabbit polymorphonuclear leucocytes contain an enzyme capable of hydrolyzing biosynthetic phenolphthalein mono-β-glucuronide. The concentration of the enzyme in the white cell is some 2000 times the concentration of the enzyme in the blood plasma. Under the conditions of study, the β-glucuronidase activity was proportional to the concentration of the enzyme. The effect of substrate concentration on the enzyme activity was studied and the Michaelis constant, K_s, determined. The course of the reaction was linear with time for the first 12 hr. and then fell off slightly during the next 12 hr. The optimum pH of the enzyme was 4.45 in either 0.2 *M* acetate or 0.2 *M* phthalate buffer. It was not inhibited by cyanide, azide, iodoacetate, fluoride, glycine, thiourea, urethane, arsanilic acid, acetophenone, *o*-cresol or *m*-cresol, in a final concentration of 0.01 *M*. The possible function of β-glucuronidase in rabbit polymorphonuclear leucocytes is discussed.

Introduction

Rabbit polymorphonuclear leucocytes contain a phosphomonoesterase (Cram and Rossiter (1, 2)) and an ali- or common esterase (Rossiter and Wong (35, 36)). It has now been shown that these cells also contain a β-glucuronidase capable of hydrolyzing phenolphthalein mono-β-glucuronide.

In 1914 Sera (38) showed that the liver, kidney, and spleen of the ox, rabbit, and dog contain an enzyme that is able to hydrolyze glucuronides. Subsequently the enzyme was partially purified by Masamune (26), Oshima (32), and others (4, 10, 12, 17). In 1946 Fishman introduced a convenient method for determining the activity of β-glucuronidase, based on the liberation of free phenolphthalein, colored at pH greater than 10, from biosynthetic phenolphthalein mono-β-glucuronide (39). This determination and the method of preparation of the substrate was later improved (9).

Fishman, Springer, and Brunetti (9) reported that there was considerable β-glucuronidase activity in human blood and that much of this was in the buffy-coat layer. Rossiter and Wong (37) showed that for man the enzyme was present in both the polymorphonuclear leucocyte and the lymphocyte, the ratio being of the order of 5 : 4. In the present paper, some of the properties of the β-glucuronidase of rabbit polymorphonuclear leucocytes are described.

[1] *Manuscript received November 14, 1949.*

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Methods

Cell Preparation

Polymorphonuclear leucocytes were obtained from the rabbit by the method of de Haan (13), the details of which have already been described by Cram and Rossiter (2). At least 95% of the cells were polymorphonuclear leucocytes, the remainder being lymphocytes or monocytes.

β-Glucuronidase Determination

The glucuronidase activity was determined by the method of Fishman *et al.* (9) using the substrate prepared as described by these workers. To a centrifuge tube was added:—

0.2 ml. cell suspension
0.8 ml. 0.1 *M* acetate buffer, pH 4.5
0.1 ml. 0.01 *M* phenolphthalein mono-β-glucuronide
0.2 ml. 0.5% saponin.

The tube was placed in a 38° C. water bath for 2–16 hr. at the end of which time the reaction was stopped by the addition of 1 ml. 10% trichloroacetic acid. A control tube containing enzyme, buffer, and saponin was incubated for the same length of time and the substrate was added just before the trichloroacetic acid. The determination was completed as described by Fishman *et al.* (9), the test and control being read against a blank in a Coleman universal spectrophotometer at 540 mμ. A standard containing 20 μgm. phenolphthalein per tube was read with each set of determinations. The test was always run in duplicate.

The object of the saponin was to extract the enzyme from the cell. Rossiter (33) showed that certain surface-active substances, such as saponin, alkyl sulphate, or bile salts, liberate the enzymes phosphomonoesterase and aliesterase from rabbit polymorphonuclear leucocytes into the surrounding fluid. It is shown in this paper that saponin also liberates β-glucuronidase from these cells.

Recording of Results

Enzyme activity has been recorded in terms of glucuronidase units per 100 ml. cell suspension where, following Fishman (9, 39), one glucuronidase unit is defined as the amount of enzyme that would liberate one μgm. phenolphthalein in one hour under the standard conditions of the test. To bring the values for white-cell glucuronidase into line with those of other white-cell enzymes studied in the laboratory, the results have also been given in terms of glucuronidase units per 10^{10} cells. This has the further advantage that 10^{10} packed cells has a wet weight in the neighborhood of 4 gm. (34). It is thus possible to compare the concentration of the enzyme in the leucocyte with that in other tissues.

Results

Concentration of β-Glucuronidase in Rabbit Polymorphonuclear Leucocytes

Table I gives the concentration of β-glucuronidase in rabbit polymorphonuclear leucocytes and Table II gives the concentration of the enzyme in

TABLE I

β-GLUCURONIDASE ACTIVITY OF SUSPENSIONS OF RABBIT POLYMORPHONUCLEAR LEUCOCYTES

Rabbit No.	Units/10^{10} cells	Units/100 gm. tissue
1	8600	215,000
2	13,300	332,000
3	21,100	527,000
4	10,800	270,000
5	9900	248,000
6	11,100	277,000
7	9100	238,000
8	9100	238,000
9	8900	223,000
10	11,800	295,000
14	4600	115,000
15	7700	192,000
16	5600	140,000
17	6500	165,000
18	7900	198,000
Mean (± S.D.) =	9700 (± 3800)	243,000 (± 94,000)

TABLE II

β-GLUCURONIDASE ACTIVITY OF RABBIT PLASMA

Rabbit No.	Units/100 ml.	Rabbit No.	Units/100 ml.
1	127	5	67
2	131	12 R2	67
3	113	13 RB1	226
4	117	8 RX	76
5 R1	203	9 RX	9

Mean (± S.D.) = 114 ± 62

rabbit plasma. The mean (±S.D.) enzyme activity of the white cells was 9700 (± 3800) units per 10^{10} cell or 243,000 units per 100 gm. tissue. The mean activity of the white cells was thus some 2000 times greater than that of the rabbit plasma, 114 (± 62) units per 100 ml.

The Extraction of Enzyme from Cell

Fishman *et al.* (9) extracted β-glucuronidase from white blood cells by the alternate freezing and thawing of the cells. Since it had previously been shown that other enzymes could be extracted by surface-active substances (33), the effect of adding saponin to suspensions of rabbit polymorphonuclear leucocytes was investigated (Table III). One sample of cells was suspended in isotonic saline and a similar sample in 0.5% saponin in isotonic saline. After the β-glucuronidase activity had been determined in each, the two suspensions were centrifuged. The resulting cell-containing residues were made up to

their original volume with isotonic saline. The β-glucuronidase activity of each of the two residues and each of the two cell-free supernatants was then determined.

The first column of Table III shows that when saponin was added to the cells the activity of the suspension was greater. A similar finding was reported for phosphomonoesterase (2, 33). Presumably, because of the limited permeability of the cell membrane to phenolphthalein mono-β-glucuronide, the substrate did not saturate all the active centers of the enzyme within the cell. When saponin was added, substrate was available to all of the enzyme and maximum activity resulted. That the enzyme was liberated from the cell is shown by the second and third columns of Table III. For the cells originally

TABLE III

EFFECT OF 0.5% SAPONIN ON THE DISTRIBUTION OF β-GLUCURONIDASE BETWEEN CELLS AND SUPERNATANT OF SUSPENSIONS OF WASHED RABBIT POLYMORPHONUCLEAR LEUCOCYTES

Cells suspended in	Glucuronidase activity (units/100 ml.)		
	Original suspension	Supernatant	Cells
Isotonic saline	244	34	185
0.5% saponin in isotonic saline	266	249	13
Isotonic saline. Cells centrifuged off and resuspended in 0.5% saponin in isotonic saline	—	—	244

suspended in isotonic saline, the greater part of the enzyme activity, 185 units per 100 ml., remained in the cell-containing residue and only a little, 34 units per 100 ml., was in the supernatant. For the cells originally suspended in 0.5% saponin in isotonic saline, the greater part of the activity, 249 units per 100 ml., was in the cell-free supernatant, only 13 units per 100 ml. remaining in the cells. Moreover, when 0.5% saponin in isotonic saline was added to cells that previously had been suspended in saline only and separated by centrifuging, the activity was increased from 185 units per 100 ml. to 244 units per 100 ml., an increase similar to that observed when saponin was added to the original suspension.

The effect of adding 0.2 ml. 0.5% saponin to the reaction mixture containing 0.2 ml. cell suspension was compared with the freezing–thawing method in the experiments reported in Table IV. Although freezing and thawing did increase the enzyme activity of a suspension of cells in water, the effect of adding 0.2 ml. 0.5% saponin to the reaction mixture was much greater. The addition of saponin to the cells that previously had been frozen and thawed produced little more activity than did the addition of saponin to cells that had not been subjected to the freezing–thawing procedure.

TABLE IV

COMPARATIVE EFFECTS OF SAPONIN AND ALTERNATE FREEZING AND THAWING ON THE β-GLUCURONIDASE ACTIVITY OF SUSPENSIONS OF RABBIT POLYMORPHONUCLEAR LEUCOCYTES

Additions to reaction mixture	Glucuronidase activity (units/100 ml.)	
	Preparation I	Preparation II
0.2 ml. water	614	279
0.2 ml. 0.5% saponin	**935**	**353**
0.2 ml. water cells frozen and thawed nine times	746	329
0.2 ml. 0.5% saponin cells frozen and thawed nine times	990	347

Table V shows that, for the cell suspensions used, 0.2 ml. 0.5% saponin produced a maximal effect. Concentrations of saponin greater than 1% were slightly inhibitory, while concentrations less than 0.5% were not sufficient to liberate all the enzyme from the cells.

TABLE V

EFFECT OF SAPONIN ON THE β-GLUCURONIDASE ACTIVITY OF SUSPENSIONS OF RABBIT POLYMORPHONUCLEAR LEUCOCYTES

Additions to reaction mixture	Glucuronidase activity (units/100 ml.)	
	Preparation I	Preparation II
0.2 ml. water	350	536
0.2 ml. 10% saponin	338	518
0.2 ml. 5% saponin		539
0.2 ml. 1% saponin	412	575
0.2 ml. 0.5% saponin	**423**	**592**
0.2 ml. 0.1% saponin	417	560
0.2 ml. 0.01% saponin	—	508

Sodium taurocholate (10^{-3} *M*) had an effect similar to that of saponin.

Enzyme Concentration

Under the conditions of study, the β-glucuronidase activity of a preparation of rabbit polymorphonuclear leucocytes was proportional to the concentration of the enzyme (Fig. 1).

Substrate Concentration

Fig. 2 shows the effect of the concentration of the substrate on the hydrolysis of phenolphthalein glucuronide by the rabbit polymorphonuclear-leucocyte enzyme. The curve is the typical hyperbola of an enzyme whose enzyme–substrate relation can be described in terms of Michaelis–Menten theory. Substrate concentrations of 0.001 *M* or above produced maximum enzyme activity. In contrast to the findings of Talalay *et al.* (39) for a partially

purified enzyme preparation from mouse spleen, liver, and kidney, excess of the substrate was not inhibitory for substrate concentrations up to 0.0022 *M*. Higher substrate concentrations were not tested. With other substrates and

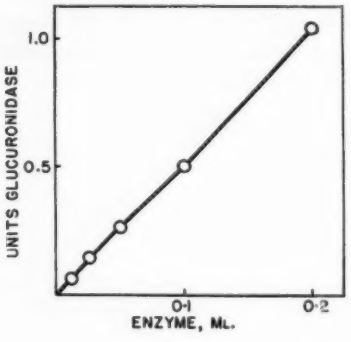

FIG. 1

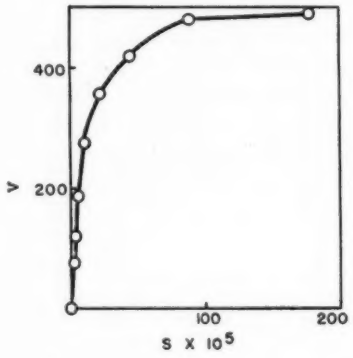

FIG. 2.

FIG. 1. *The relation between enzyme activity and enzyme concentration of rabbit polymorphonuclear leucocyte β-glucuronidase.*

FIG. 2. *The effect of substrate concentration on the initial velocity of rabbit polymorphonuclear leucocyte β-glucuronidase. V is the initial velocity in mgm. phenolphthalein per 100 ml. per hr. and S is the substrate concentration in gm. molecules per liter. Substrate, phenolphthalein mono-β-glucuronide. Temperature, 38° C.*

other enzyme sources, inhibition of β-glucuronidase by excess substrate has been described (5, 18).

Fig. 3 is a plot of $1/V$ against $1/S$ according to Lineweaver and Burk (24). The Michaelis constant, K_s, of four different preparations of the enzyme from

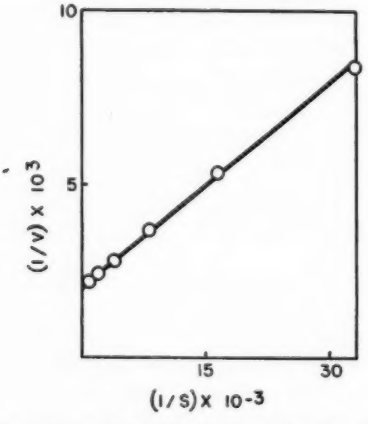

FIG. 3. *Plot of 1/V against 1/S for rabbit polymorphonuclear leucocyte β-glucuronidase. V is the initial velocity in mgm. phenolphthalein per 100 ml. per hr. and S is the substrate concentration in gm. molecules per liter.*

polymorphonuclear leucocytes was 0.000093 *M*, 0.000100 *M*, 0.000111 *M*, and 0.000117 *M*, respectively. These figures are slightly higher than the values of 0.000053 *M* and 0.000045 *M* for the K_s of the β-glucuronidase from mixed mouse organs reported by Talalay *et al.* (39) using the same substrate, buffer, and hydrogen ion concentration. The values of K_s for phenolphthalein glucuronide are much lower than those reported in the literature for other substrates, 0.0005 *M* for oestriol glucuronide, 0.004 *M* for menthyl glucuronide, 0.01 *M* for borneol glucuronide with ox-spleen β-glucuronidase (5), and 0.0035 *M* for phenyl glucuronide with mouse-liver enzyme (18).

Course of Reaction with Time

Under the conditions of study, the rate of hydrolysis of phenolphthalein glucuronide was reasonably constant with time for the first 12 hr. During the second 12 hr., the decrease in activity was slight (Fig. 4). Table VI shows that if the glucuronidase activity of an enzyme preparation is determined using incubation periods up to 12 hr. the results are satisfactory. The result for a 24-hr. incubation period is some 10% too low.

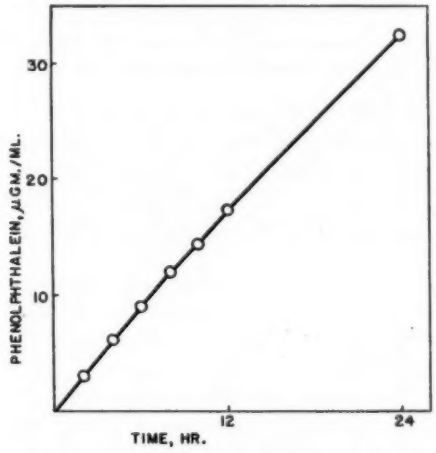

FIG. 4. *Time course of the reaction for rabbit polymorphonuclear leucocyte β-glucuronidase. Substrate, phenolphthalein mono-β-glucuronide. Temperature, 38° C.*

Hydrogen Ion Concentration

The optimum hydrogen ion concentration was 4.45 in 0.2 *M* acetate buffer (Fig. 5). A similar figure was obtained when 0.2 *M* phthalate buffer was used. This agrees with the values found by Talalay *et al.* (39) using phenolphthalein glucuronide as substrate, although other workers employing different substrates and different enzyme sources have reported pH optima varying from 4.3 to 5.6 (5, 18, 26, 32). Mills (28) separated ox-spleen β-glucuronidase into two fractions, one having an optimum pH of 4.5 for menthyl, phenyl, and phenolphthalein glucuronide and one having an optimum pH of 5.0–5.2 for

the same three substrates. Fig. 5 shows that in the preparation used, there was no suggestion of a peak at pH 5.0–5.2. Kerr *et al.* (18) reported that the β-glucuronidase of mouse liver and spleen also had two pH optima, one in

TABLE VI

COURSE OF REACTION WITH TIME. ENZYME, β-GLUCURONIDASE OF RABBIT POLYMORPHONUCLEAR LEUCOCYTES, SUBSTRATE, PHENOLPHTHALEIN MONO-β-GLUCURONIDE

TEMPERATURE, 38° C.

Time (hr.)	Phenolphthalein liberated (μgm./ml. reaction mixture)	Glucuronidase activity (units/10^{10} cells)
2	3.0	970
4	6.1	990
6	9.0	970
8	12.0	970
10	14.2	920
12	17.3	930
24	32.3	880

the region of pH 4.5 and one in the region of pH 5.2. Subsequently these workers showed (17) that the same was true for mouse-kidney β-glucuronidase, but not for the enzyme from the mouse uterus. It would thus appear that, on the basis of the pH-activity curve, the β-glucuronidase of rabbit polymorphonuclear leucocytes resembles that of the mouse uterus.

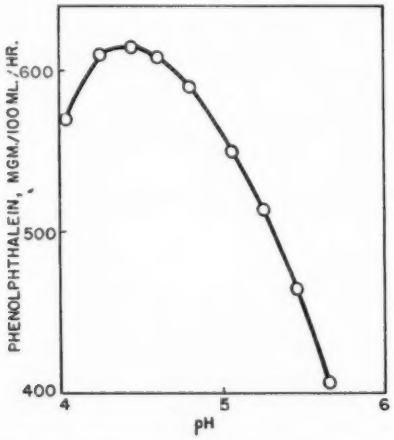

FIG. 5. *The effect of hydrogen ion concentration on the activity of rabbit polymorphonuclear leucocyte β-glucuronidase. Substrate phenolphthalein mono-β-glucuronide. Temperature, 38° C. Buffer, 0.2 M acetate.*

Inhibitors

Glucuronidase is not inhibited by many of the usual enzyme inhibitors. The following substances produced negligible inhibition when present in the reaction mixture in a final concentration of 0.01 *M*: cyanide, azide, iodoacetate, fluoride, glycine, thiourea, urethane, arsanilic acid, acetophenone, *o*-cresol, and *m*-cresol. Of these substances, fluoride (0.015 *M*), thiourea (0.0015 *M*), and urethane (0.015 *M*) have been shown to have no effect on mouse-liver β-glucuronidase (16).

Discussion

The distribution of β-glucuronidase in the animal body has been studied by Oshima (31) and Talalay *et al.* (39). Both groups of workers found high enzyme activity in the spleen. Because of the limited amount of material available no attempt was made to purify the enzyme from the rabbit polymorphonuclear leucocytes. If, as has now been shown for ox-spleen and other organs (17, 18, 28, 29), it were found that the rabbit polymorphonuclear leucocyte contained two or more enzymes, it is possible that the kinetic data would have to be reinterpreted. As has been pointed out above, a study of the pH-activity curve for the crude rabbit polymorphonuclear leucocyte enzyme shows little evidence of more than one maximum.

The high concentration of β-glucuronidase found in the polymorphonuclear leucocyte of the rabbit and also in the white cells of man (9, 37) raises the question of whether the white cells might not be an important source of plasma β-glucuronidase. But, as was pointed out for phosphomonoesterase (2), the possibility still remains that the white cell may have obtained its β-glucuronidase by adsorption of the enzyme from the plasma. If this were so, the white cells must have the extraordinary ability of concentrating the enzyme by a factor of 2000, for the concentration of β-glucuronidase was of the order of 250,000 units per 100 gm. packed white cells compared with 115 units per 100 ml. rabbit plasma. It seems likely that the enzyme is within the cell rather than adsorbed on the cell surface, for it has been demonstrated histochemically by Friedenwald and Becker (11) in the cells of the spleen, lymphatic nodules, and bone marrow. The enzyme was in the cytoplasm rather than in the nucleus.

It is interesting to speculate upon the function of β-glucuronidase in polymorphonuclear leucocytes; especially since it has been suggested that β-glucuronidase has to do with metabolic conjugation and, possibly, detoxication (6, 7, 31). Polymorphonuclear leucocytes quickly aggregate at sites of infection. It would be attractive to assume that here they inactivated toxic substances by conjugation. The theory that β-glucuronidase is concerned with metabolic conjugation and detoxication has, however, been severely criticized (15, 16, 21, 22). The chief function of the enzyme appears to be hydrolytic (27, 30). It is clear from the work of Levvy and his associates that the *in vitro* synthesis of glucuronides is brought about by a different

enzyme system from the β-glucuronidase that hydrolyzes biosynthetic glucuronides (21, 23). Inhibition of glucuronide synthesis does not inhibit glucuronide hydrolysis (3,14,25) nor does the reduction of glucuronide hydrolysis by either the *in vitro* (16) or the *in vivo* (15) administration of inhibitors decrease glucuronide synthesis. Also, if glucuronide hydrolysis is increased, there is no increase in glucuronide synthesis (15).

The chief proponent of the conjugation theory is Fishman. He showed that the injection of menthol to dogs and mice caused an increase in the β-glucuronidase of the liver, spleen, and kidney, with no change in the uterus (6). Subsequently he showed that the administration of oestrogens to ovariectomized mice produced an increase in the β-glucuronidase of the uterus, but no change in that of the liver (7, 8). It was suggested that the increase in the enzyme following menthol administration was evidence for the view that the enzyme has to do with the conjugation of menthol and that the increase in the uterus following oestrogen administration was evidence that it has to do with metabolic conjugation. Levvy and co-workers, on the other hand, believe that the β-glucuronidase activity of an organ parallels the degree of tissue regeneration and cell proliferation (19, 20, 22). They also suggested that the rise in the uterine β-glucuronidase after the administration of oestrogens could also be explained by cell proliferation.

If the function of β-glucuronidase is concerned with the hydrolysis of glucuronides rather than their synthesis, the role of the enzyme in the white cell may be concerned with one of the stages in the hydrolysis of a substance normally present in tissues that contain glucuronic acid e.g. hyaluronic acid or chondroitin sulphuric acid (9). It is thus conceivable that the white-cell enzyme may play some part in the complex tissue reaction of inflammation.

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USE OF OLEIC ACID – ALBUMIN AGAR MEDIUM FOR THE STUDY OF STREPTOMYCIN RESISTANCE OF *MYCOBACTERIUM TUBERCULOSIS*[1]

By Charles O. Siebenmann[2]

Abstract

The oleic acid – albumin agar, proposed by Dubos and Middlebrook as a diagnostic medium for the isolation of *Mycobacterium tuberculosis*, proved useful for determining streptomycin resistance. By growing cultures of *M. tuberculosis* on this translucent substrate, containing graded amounts of streptomycin, a close correlation was found between the observed streptomycin resistance and that determined in fluid tween-albumin medium. The initial presence of a few organisms, showing a slight resistance to streptomycin, was found not to be an exclusive characteristic of cultures developing drug resistance in the course of streptomycin therapy. Cultures not developing resistance may also contain such resistant cells.

The use of Dubos fluid medium (2) for the determination of streptomycin resistance of *Mycobacterium tuberculosis* has been adopted by many clinical laboratories as a convenient and relatively rapid method, yielding reproducible results. In the case of streptomycin-sensitive cultures, this method fails, however, to reveal the presence of streptomycin-resistant tubercle bacilli if their relative numbers are small (9). That such streptomycin-resistant cells may be present even in cultures which were never in contact with streptomycin was demonstrated for the first time by Pyle (6) who seeded such cultures on Herrold's egg yolk – agar medium containing graded amounts of streptomycin. This medium has been used successfully for determining streptomycin resistance (1, 6, 8). For investigational purposes in the field of chemotherapy, this medium has, however, some disadvantages. The presence of egg yolk makes it chemically complex and over-rich in nutrient material, which tends to mask antibacterial activity of chemotherapeutic agents to be tested. As a medium more suited for our purposes, we chose oleic acid – albumin agar, proposed by Dubos and Middlebrook (2) for the isolation of *M. tuberculosis*. Its usefulness as a diagnostic medium was confirmed by Smith *et al.* (7). It is chemically well defined and permits rapid, though not over-abundant growth. As a translucent medium, it lends itself ideally to the microscopic study of the colonial characteristics of *M. tuberculosis* grown in the presence or absence of chemo-therapeutic agents.

In the following study the use of this medium is described for the determination of streptomycin sensitivity of human strains of *M. tuberculosis*. By means of this technique, cultures of *M. tuberculosis*, isolated prior to the start of streptomycin therapy, were tested for the possible presence of streptomycin-resistant cells. The purpose of this study was to investigate whether

[1] *Manuscript received January 23, 1950.*

Contribution from the Connaught Medical Research Laboratories, University of Toronto, Toronto, Ontario.

[2] *With the technical assistance of W. H. Linklater and R. Otulakowski.*

the presence of such resistant cells could account for the later development of streptomycin resistance.

Methods

Oleic acid – albumin agar was prepared essentially according to the authors' description (2). The streptomycin was incorporated in the form of streptomycin sulphate, added as an aqueous solution, simultaneously with the oleic acid – albumin complex. This addition was made after the basal medium was cooled to 50° C. Slants were poured, using 1 oz. flat, rectangular vials with bakelite screw caps. The usable agar surface was 3 × 6 cm.

The following concentrations of streptomycin were used: 0, 0.5, 1, 10, and 100 μgm. per ml. of medium.

The growth observed on oleic acid – albumin agar was compared with the growth obtained after seeding the cultures on Loewenstein – Jensen (5) slopes contained in similar vials.

For testing cultures of *M. tuberculosis* for streptomycin sensitivity, using oleic acid – albumin agar, these were first grown for 10 days on Dubos fluid medium. For seeding the agar slants, a fivefold dilution of such cultures in Dubos medium was used. Each slant was seeded with two inocula with a 5 mm. platinum loop. Control counts, made in some instances on oleic acid – albumin agar plates, indicated that such seedings contained in the order of 50—100,000 viable cells.

Of some 20 cultures of *M. tuberculosis* seeded on these oleic acid – albumin agar slants all grew well and more rapidly, though less abundantly, than on Loewenstein–Jensen slopes, first growth generally appearing within one week. Final readings were made after six weeks. Little change was noticed after the fourth week of incubation (38–39° C.).

When using fluid Dubos medium (containing 0.05% tween 80) for determining streptomycin sensitivity the technique recommended by the Department of Veterans' Affairs* was followed in all essential features. Streptomycin sensitivity is defined as the lowest concentration of streptomycin which inhibits the growth of a culture during a 14 day period of incubation.

Presence of Partially Resistant Cells in Otherwise Streptomycin-Sensitive Cultures

Table 1 records observations made on two resistant and one partially resistant strain of *M. tuberculosis*. For comparison the three original, streptomycin-sensitive strains, from which the streptomycin-resistant variants were derived, were examined. In the first culture (H37RV) the resistance was produced by prolonged ***in vitro*** exposure of the culture to streptomycin. In the two other cultures (67 and 913) resistance developed ***in vivo*** in the course of streptomycin therapy of the patient.

* *Personal communication, courtesy of Dr. Marion Ross, Christie Street Hospital, Toronto.*

TABLE I

STREPTOMYCIN SENSITIVITY OF HUMAN STRAINS OF *M. tuberculosis*

Cultures***	Fluid Dubos medium**	Colony counts on streptomycin oleic acid agar*				
		0	0.5	1	10	100
H37RV	0.2	Innum.	0	0	0	0
H37RV Res.	>1024	Innum.		Innum.	Innum.	Innum.
15	0.2	Innum.	100	0	0	0
67 Res.	>1024	Innum.		Innum.	Innum.	Innum.
710	0.2	Innum.	6	0	0	0
913 Res.	1.0	Innum.		Innum.	0	0

* *Containing designated amounts of streptomycin expressed in μgm. per ml. of medium.*

** *Figures record minimum bacteriostatic concentrations of streptomycin in μgm. per ml.*

*** *Received from The Medical School, Northwestern University, Chicago (Courtesy of Dr. G. P. Youmans.).*

The streptomycin sensitivities of these six cultures (Table I), as determined in fluid Dubos medium (Column 2), broadly agrees with the growth observations made on oleic acid – albumin agar (Columns 3–7). In the case of streptomycin-sensitive cultures, the advantage of the solid substrate over the fluid medium becomes evident in that it reveals the presence of colonies derived from cells of increased streptomycin resistance.

To investigate whether the presence, in some cultures of *M. tuberculosis*, of a small number of resistant cells could account for the later development by these cultures of a marked drug resistance, the following series of experiments were carried out.

Two groups of pretreatment cultures of *M. tuberculosis* were examined for the presence of cells of increased streptomycin resistance:

Group A comprised of cultures obtained from patients from whom, after streptomycin therapy, drug-resistant cultures were isolated.

Group B cultures isolated from patients from whom, after streptomycin treatment, only streptomycin-sensitive cultures were obtained.

For these two groups of cultures streptomycin sensitivity was determined on oleic acid – albumin agar as described above. The results (see Table II) show that cultures containing cells of partial streptomycin resistance are found in both groups of cultures, though they are more frequent among the group which developed drug resistance in the course of streptomycin therapy. In this latter group all five cultures showed colonies in the presence of 0.5 μgm. of streptomycin, whereas in the group which remained resistant only four out of eight cultures contained such cells.

Discussion and Conclusions

From the experimental data presented it seems evident that in the case of a culture of *M. tuberculosis*, isolated prior to the start of streptomycin treatment, the presence of a few cells showing increased streptomycin resistance

TABLE II

STREPTOMYCIN SENSITIVITY OF HUMAN STRAINS OF *M. tuberculosis* ISOLATED PRIOR TO STREPTOMYCIN TREATMENT

Culture**	Growth on streptomycin oleic acid agar*					Remarks
	0	0.5	1	10	100	
B 1715	Innum.†	50	0	0	0	Became resistant
B 5017	Innum.	30	0	0	0	" "
B 5709	Innum.	Num.‡	7	0	0	" "
710	Innum.	6	0	0	0	" "
15	Innum.	Num.	0	0	0	" "
H37RV***	Innum.	Num.	0	0	0	" "
B 4240	Innum.	Num.	0	0	0	Remained sensitive
B 2611	Innum.	0	0	0	0	" "
B 6085	Innum.	0	0	0	0	" "
B 5362	Innum.	1	0	0	0	" "
B 1401	Innum.	0	0	0	0	" "
B 5862	Innum.	Num.	75	0	0	" "
B 8886	Num.	30	30	20	0	" "
B 7986	Innum.	0	0	0	0	" "

* *Containing designated amounts of streptomycin expressed in μgm. per ml. of medium.*

** *Cultures marked B were obtained from Toronto Hospital of Tuberculosis (courtesy of Dr. W. Anderson) where resistance is defined as "growth in concentrations of 50 μgm. of streptomycin per ml. on Herrold's medium" (6).*

*** *Included as example of strain capable of developing streptomycin resistance in vitro.*

† *"Innum." indicates abundant growth with innumerable colonies present.*

‡ *"Num." indicates presence of from 100 to approximately 500 colonies.*

cannot serve as a sure prognostic indication of early development of drug resistance. This is illustrated by the cultures B5862 and B8886, which despite the presence of partially resistant cells, did not develop drug resistance in the course of streptomycin therapy.

Although the initial presence of partially resistant cells may be of significance for the later development of streptomycin resistance, there must be other factors at work which determine the emergence of resistant strains. In this connection, recent clinical reports (3, 4) are of special interest which indicate that streptomycin resistance is encountered predominantly among cases of pulmonary tuberculosis in which, prior to streptomycin treatment, definite caseation or cavitation was present.

The difficulty of predicting, by means of a simple laboratory test, the later development of streptomycin resistance, should not detract from the importance of testing the initial cultures by means of solid media for the presence of streptomycin-resistant cells. In view of the increasing possibility of incoming patients having contracted the infection from carriers of resistant strains, the careful testing of such cultures for their streptomycin sensitivity is a matter of considerable urgency. For such strains of *M. tuberculosis*, even if only partially drug-resistant, may present serious problems which will be the subject of a subsequent communication.

Acknowledgments

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THE EFFECTS OF SOME URINARY EXTRACTS ON GASTRIC SECRETION IN THE SHAY OPERATED RAT[1]

By Floyd R. Skelton and Gordon A. Grant

Abstract

Following the general procedure of Risley, Raymond, and Barnes the effects of urinary extracts on gastric secretion have been studied in the Shay operated young, male, albino rat. The procedure consisted of ligating the pylorus following a 24 hr. fast. Immediately postoperatively the test material was administered. After a five hour period the stomach was removed, opened, and rinsed into 10 ml. of distilled water. An aliquot of this was titrated for its free and total acid content. Human chorionic gonadotrophin and preparations obtained by the benzoic acid absorption method from human and equine pregnancy urine were studied for their effects on gastric section using the above technique after subcutaneous, intraduodenal, and oral administration. It was found that chorionic gonadotrophin caused gastric secretory depression at a dose level as low as 10 mgm. per rat subcutaneously. Both the human and equine preparations were observed to reduce the gastric secretion proportionately to the dose when administered subcutaneously. When administered by intraduodenal injection at the time of operation, the equine preparation was active but required several times the subcutaneous dose to produce comparable results. Oral administration of both equine and human pregnancy urine extracts showed antisecretory activity only in the former at the dose levels employed. In addition, the effects of possible complicating factors such as estrogens, pyrogens, and nonspecific damage on gastric secretion have been investigated and found to play no appreciable part in the results produced by the urine fractions.

Introduction

There is convincing evidence that the intestinal mucosa produces a substance "Enterogastrone" which passes into the circulation and inhibits the secretion of gastric juice (2, 3, 6, 13, 14, 15, 10). A similar effect of various urine extracts on gastric secretion has more recently been reported by many investigators (17, 4, 7, 12). The active substance has been called "Urogastrone" and whether it is a separate entity from enterogastrone is not yet certain (5, 8, 9, 11, 20). The reduction in gastric acidity and in the incidence of peptic ulceration during pregnancy (1, 2, 22) has suggested the possibility of the production of an "anti-secretory" or "anti-ulcer" principle at this time. Consequently studies of pregnancy urine as a possible source of such a substance have been carried on by numerous laboratories. The inhibition of ulceration in the Shay operated rat by extracts of intestine and urine is in general accompanied by a roughly parallel decrease in the volume and acid concentration of the gastric juice. On this basis, Risley, Raymond, and Barnes (18) have recently shown that the measurement of gastric secretory inhibition in the Shay operated rat can be used as an indication of the antisecretory activity of such extracts. The purpose of this communication is to present additional data on the use of the Shay operated rat in an antisecretory assay and to report the effects of some pregnancy urine preparations as well as some nonspecific procedures on the gastric secretory response.

[1] *Manuscript received February 15, 1950.*

Contribution from the Research and Biological Laboratories, Ayerst, McKenna, and Harrison, Ltd. Montreal, Canada.

Materials and Methods

The method employed in this study was essentially the same as that of Risley *et al.* but was adapted to male, albino rats 30 days of age and weighing 50–60 gm. Following a 24 hr. fast the pylorus was ligated under light ether anesthesia and immediately postoperatively the substance to be tested was administered. After an interval of five hours during which time no food or water was allowed, the stomach was removed, opened, and rinsed into 10 ml. of distilled water. The volume of gastric secretion was determined by subtraction and an aliquot was taken for titration with $N/100$ sodium hydroxide to determine the free and total acid of the gastric contents. Finally, the free and total acid in the contents of the stomach was expressed as ml. of $N/100$ hydrochloric acid.

The results of all extracts assayed were evaluated in comparison with simultaneously conducted untreated control groups. Each group consisted of 10 or more animals.

Results

The experimental observations are presented in the following tables. The expressed values include the standard error of the mean.

TABLE I

EFFECT OF SUBCUTANEOUS ADMINISTRATION OF HUMAN PREGNANCY URINE EXTRACTS ON GASTRIC SECRETION IN THE RAT

Treatment	Dosage in mgm. per rat	Ml. of gastric secretion	Ml. of $N/100$ HCl in gastric contents	
			Free acid	Total acid
Control		2.0 ± 0.27	11.8 ± 1.5	20.3 ± 2.5
Chorionic gonadotrophin	10	1.2 ± 0.31	2.0 ± 0.78	7.6 ± 2.4
Benzoic acid adsorbate of human pregnancy urine	12	0.5 ± 0.07	0	1.8 ± 0.46
	6	0.6 ± 0.12	0	2.8 ± 1.1
	2	0.7 ± 0.11	0.9 ± 0.43	4.3 ± 0.75

Table I illustrates the effects of human pregnancy urine extracts on gastric secretion after subcutaneous injection. A 10 mgm. dose of chorionic gonadotrophin (APL) caused marked reduction in volume and acidity of gastric juice. The benzoic acid adsorbate fraction represents the residual urine extract after the chorionic gonadotrophin has been removed. It can be seen that it was much more active than APL in depressing gastric secretory activity and that there was a general dose–response relationship.

In Table II the results of the subcutaneous administration of a benzoic acid adsorbate from pregnant mare's urine are outlined. This preparation was also highly active and appears to have a similar dose–response relationship.

TABLE II

EFFECT OF SUBCUTANEOUS ADMINISTRATION OF AN EXTRACT FROM PREGNANT MARE'S URINE ON GASTRIC SECRETION IN THE RAT

Dosage in mgm. per rat	Ml. of gastric secretion	Ml. of *N*/100 HCl in gastric contents	
		Free acid	Total acid
Control	2.2 ± 0.2	15.7 ± 2.0	26.6 ± 2.4
100	0	0	1.9 ± 0.3
50	0.1 ± 0.05	0.2 ± 0.1	3.0 ± 0.4
10	2.1 ± 0.05	13.7 ± 2.2	24.6 ± 1.7

The effect of intraduodenal administration of the benzoic acid adsorbate preparations from both human and equine pregnancy urine has been investigated. Table III shows that following intraduodenal injection the human urine extract caused only slight secretory depression without any apparent dose–response relationship. Table IV shows a progressively increasing secretory inhibition with increasing doses of mare's urine extract. However,

TABLE III

EFFECT OF INTRADUODENAL ADMINISTRATION OF A BENZOIC ACID ADSORBATE OF HUMAN PREGNANCY URINE ON GASTRIC SECRETION IN THE RAT

Dosage in mgm. per rat	Ml. of gastric secretion	Ml. of *N*/100 HCl in gastric contents	
		Free acid	Total acid
Control	3.2 ± 0.14	24.8 ± 2.0	43.3 ± 2.6
50	2.3 ± 0.2	7.9 ± 2.4	38.8 ± 4.0
30	1.8 ± 0.3	8.0 ± 1.8	20.3 ± 2.6
10	1.8 ± 0.2	10.0 ± 1.8	20.3 ± 2.7

TABLE IV

EFFECT OF INTRADUODENAL ADMINISTRATION OF AN EXTRACT FROM PREGNANT MARE'S URINE ON GASTRIC SECRETION IN THE RAT

Dosage in mgm. per rat	Ml. of gastric secretion	Ml. of *N*/100 HCl in gastric contents	
		Free acid	Total acid
Control	3.3 ± 0.14	27.4 ± 1.6	40.6 ± 1.8
400	0	0	1.7 ± 0.8
200	0.3 ± 0.09	1.1 ± 0.6	6.1 ± 1.2
100	1.7 ± 0.2	9.8 ± 1.8	20.6 ± 2.3
50	2.6 ± 0.3	19.4 ± 3.6	33.8 ± 4.2

approximately eight times the effective subcutaneous dose of equine urine extract was necessary to obtain an equivalent result.

Oral administration of the urine preparations was effected in the following manner. The extract was given by gavage in equally divided doses, 24 and 16 hr. prior to pyloric ligation to ensure complete absorption. It was found that under these conditions a dose of the human pregnancy urine extract 16 times the effective subcutaneous dose had no effect on gastric secretion (Table V). In contrast a marked depression of volume and acidity of gastric juice was obtained with the equine pregnancy urine extract in a dose only nine times the effective subcutaneous dose (Table VI).

TABLE V

EFFECT OF ORAL ADMINISTRATION OF A HUMAN PREGNANCY URINE EXTRACT ON GASTRIC SECRETION IN THE RAT

Dosage in mgm. per rat	Ml. of gastric secretion	Ml. of $N/100$ HCl in gastric contents	
		Free acid	Total acid
Control	2.4 ± 0.13	11.0 ± 1.1	23.8 ± 1.6
100	2.3 ± 0.18	12.4 ± 1.6	22.4 ± 1.7

TABLE VI

EFFECT OF ORAL ADMINISTRATION OF AN EQUINE PREGNANCY URINE EXTRACT ON GASTRIC SECRETION IN THE RAT

Dosage in mgm. per rat	Ml. of gastric secretion	Ml. of $N/100$ HCl in gastric contents	
		Free acid	Total acid
Control	3.4 ± 0.19	27.6 ± 2.5	39.2 ± 2.9
450	1.6 ± 0.16	4.8 ± 0.7	15.1 ± 1.3

To exclude the possibility that some secondary complicating factor would explain the above findings, the effects of the substances and procedures shown in Table VII were investigated. As the urine extracts were not totally estrogen free it was necessary to determine the effects of this hormone on gastric secretion. A dose of sodium estrone sulphate five times greater than the amount present in the largest subcutaneous dose of pregnant mare's urine extract was required to cause any significant depression of gastric secretion and acidity. Consequently it was considered that the estrogen content of the extracts did not explain the previous results. McGinty *et al.* have recently reported some inhibition of ulcer formation in Shay operated rats following intravenous and intraperitoneal injections of bacterial pyrogen (20). Although the mare's urine preparation was substantially free of pyrogens, the human pregnancy urine extract (benzoic acid adsorbate after removal of chorionic

TABLE VII

EFFECTS ON GASTRIC SECRETION OF SOME INTERFERING SUBSTANCES AND NONSPECIFIC PROCEDURES IN THE SHAY OPERATED RAT

Treatment	Dosage in mgm. per rat	Ml. of gastric secretion	Ml. of $N/100$ HCl in gastric contents	
			Free acid	Total acid
Control	5	3.2 ± 0.13	30.5 ± 1.9	46.9 ± 2.2
Sodium estrone sulphate		1.6 ± 0.17	15.4 ± 2.5	24.5 ± 2.8
Pyrogen*	0.1	3.2 ± 0.05	32.6 ± 2.0	47.8 ± 1.8
Intestinal trauma	0.1 Ml.	1.1 ± 0.13	7.1 ± 1.5	17.9 ± 1.9
10% Formalin		1.7 ± 0.38	14.3 ± 3.5	29.1 ± 6.5
Cervical cord transection		0.5 ± 0.29	4.8 ± 1.6	9.3 ± 2.9

* *Pure Microbial Pyrogen. Baxter Laboratories Inc. Morton Grove, Ill.*

gonadotrophin) was known to be high in pyrogenic activity. For these reasons it was necessary to investigate the effect of subcutaneous pyrogen administration on gastric secretion under our experimental conditions. It can be seen in the table that at the dose level employed bacterial pyrogens did not show antisecretory activity. The effect of nonspecific stress on gastric secretion was investigated as well, because of the possibility that toxicity of the extracts might explain their effects. While the three types of stress did cause reduction in gastric secretion and acidity, the effect was not comparable in degree to that obtained with the urinary extracts although the animals were obviously more damaged.

Discussion

The decreased gastric secretion produced by chorionic gonadotrophin (APL) from human pregnancy urine confirms previous reports by Sandweiss (19), Culmer, Atkinson, and Ivy (4), and Broad and Berman (3), who observed a similar effect upon gastric secretion in the dog. The fact that the residual urine fraction is much more potent in antisecretory activity than APL suggests that this action of the gonadotrophin may be due to the presence of antisecretory substances. This was confirmed by heating the APL, a procedure which destroyed the gonadotrophin activity but not the antisecretory activity.

The extracts from human and equine pregnancy urine with antisecretory effects apparently have fundamental differences. Both are active when administered subcutaneously and both have a rough dose–response relationship. However, when administered intraduodenally and orally the preparation from equine urine had greater antisecretory activity. This is to our knowledge the first report of an orally active antisecretory extract from urine.

The possibility that the estrogen content of our extracts was the causative factor was eliminated. A dose of sodium estrone sulphate larger than the

amount of estrogens in the largest subcutaneous dose of urine extracts produced only slight secretory depression.

Intravenous and intraperitoneal administration of purified bacterial pyrogen in doses of 15–30 μgm. and 100–1000 μgm., respectively, cause gastric secretory depression as well as a reduction of ulcer incidence in the Shay rats (20). Oral administration was without effect. When administered subcutaneously in our experiments, 100 μgm. of pyrogen had no effect on the secretion of gastric juice.

The depression of gastric secretion due to nonspecific damage is of interest because of the gastric erosions which are known to occur from exposure to stress (21). However, as the stress necessary to produce such an effect was far more than that caused by treatment with the extracts it apparently does not explain our observations.

Summary and Conclusion

The antisecretory properties of chorionic gonadotrophin (APL) and of preparations obtained by the benzoic acid adsorption method from human and equine pregnancy urine were studied in the Shay operated rat following subcutaneous, intraduodenal, and oral administrations. All three urinary preparations produced gastric secretory depression when administered subcutaneously. Oral treatment with the human and equine urine fractions revealed appreciable depressant effects on gastric secretion and acidity by the equine urine preparation only. When injected intraduodenally at the time of pyloric ligation both human and equine urine extracts were active, the latter showing a definite dose–response relationship.

Bacterial pyrogens were ineffective at the levels employed in altering gastric secretory activity when administered subcutaneously.

Large doses of sodium estrone sulphate as well as severe nonspecific damage caused some reduction of gastric secretion and acidity but insufficient to explain the results obtained with the urinary extracts investigated.

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A COMPARISON OF STERNAL AND SPINOUS PROCESS MARROW[1]

By R. A. Polson and Evelyn A. Packham

Abstract

Confirming previous reports, a cytological study of the spinous process and sternal marrow showed a marked similarity in two groups of normal subjects. In each of 12 patients with various hematopoietic disorders samples were taken from the sternum and the spinous process. The marrows from the two sites were nearly identical. These findings suggest that the present knowledge of changes occurring in sternal marrow in disease may be applied to spinous process marrow.

Introduction

Early in 1948 Huss *et al.* (1) and Loge (2) described a technique for obtaining samples of marrow from the spinous processes of lumbar vertebrae. Their reports suggest that the procedure is easier and more convenient than sternal puncture, and that in subjects without gross abnormalities of the marrow, samples from the two sites are essentially similar. The purpose of the present report is to confirm these findings and to show that a close resemblance exists between marrow from these two sites even in conditions characterized by wide deviations from the normal.

Methods

The sites chosen for marrow aspiration were the sternum and the spinous process of the second or third lumbar vertebra. All sternal punctures were performed using a straight Osgood needle.

Spinous process punctures were performed by the method described by Loge (2). The patient either lay on his side or sat with his back flexed. After preparation of the area with iodine and alcohol, the skin, subcutaneous tissues, and periosteum over the selected spinous process were infiltrated with 2% novocaine. A straight Osgood marrow puncture needle was then introduced directly over the spinous process. The cortical bone was perforated with a rotatory motion of the needle and the narrow cavity entered. A well-fitting dry syringe was attached to the needle and 0.5 ml. of marrow were withdrawn by a brisk pull on the plunger. This maneuver seemed to reduce the admixture of blood in the marrow specimen. Differential cell counts on 500 cells were done on each specimen and total nucleated cell counts per cubic millimeter were performed in most instances.

Results

Sternal marrow aspirations were performed on one group of 11 healthy laboratory technicians and internes, and spinous process aspirations on

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another group of 12 similar subjects. The results are summarized in Table I, which shows the range and mean values for each type of cell. It will be seen that the marrows from the two sites showed a marked similarity.

TABLE I

DIFFERENTIAL CELL COUNTS OF THE STERNAL AND SPINOUS PROCESS MARROW OF NORMAL SUBJECTS

Cell type	Spine, 12 subjects		Sternum, 11 subjects	
	Mean	Range	Mean	Range
Blasts	0.8	0.4–1.6	0.4	0.2–1.0
Promyelocytes	3.9	2.2–6.6	3.4	2.6–4.8
Myelocytes	6.8	4.0–9.6	7.3	3.2–8.6
Metamyelocytes	6.7	5.0–11.8	6.8	3.8–9.0
Polymorphonuclears, young	20.3	12.6–27.8	18.1	13.0–22.0
Polymorphonuclears, mature	23.8	18.6–30.4	19.4	13.6–22.6
Eosinophiles	3.4	1.0–5.0	3.2	1.2–5.4
Basophiles	0.4	0–1.0	0.3	0–1.2
Plasma cells	0.3	0–1.2	0.1	0–0.8
Disintegrated	7.6	3.0–15.0	8.5	4.4–11.6
Unclassified	0	0–0.4	0	0–0.2
Lymphocytes	12.1	6.6–22.0	14.5	10.0–23.0
Lymphocytes, immature	0	0–0	0	0–0
Monocytes	2.8	1.0–4.6	2.5	1.0–3.8
Monocytes, immature	0	0–0	0	0–0
Megaloblasts	0	0–0.2	0.1	0–0.6
Erythroblasts	4.6	0.8–9.0	6.0	3.4–8.2
Normoblasts	6.7	2.0–13.4	7.2	6.0–14.6
Nucleated cells per cu. mm. marrow (in thousands)	45	19–75	45	18–100

Simultaneous sternal and spinous process punctures were performed on 13 patients with various hemopoietic diseases. The results are shown in Tables II and III. In Table II it will be seen that in six patients with lymphocytic leukemia the pathognomonic increase in the lymphocytic series was found in both types of marrow, and the degree of abnormality showed a striking correspondence. A similar correspondence was found in the counts of the granulocytic series of cells in the two cases of myelocytic leukemia reported in Table III. In the four cases of secondary anemia also reported in Table III it will be seen that moderately large variations were encountered, particularly in the counts of the erythroblasts. However, the agreement is as close as that found by Reich and Kolb (3), who compared marrows taken from different parts of the sternum. In one case of pernicious anemia, the cell counts of the sternal and spinous process marrow showed a marked similarity in the increase in megaloblasts which characterize this disease.

Each patient who underwent both sternal and spinous process marrow puncture was questioned as to his preference. In the great majority of cases, the spinous process procedure was preferred. In a small number no difference in discomfort was noted, and in no case was sternal puncture preferred.

TABLE II

DIFFERENTIAL CELL COUNTS OF THE STERNAL AND SPINOUS PROCESS MARROW OF PATIENTS WITH LYMPHOCYTIC LEUKEMIA

Cell type	Case No.											
	1		2		3		4		5		6	
	Source											
	SP*	ST**	SP	ST	SP	ST	SP	ST	SP	ST	SP	ST
Blasts	0	0	0	0	0	0	0.2	0.6	0.2	0.8	0.8	2.8
Promyelocytes	0.2	2.6	0	0	0.3	0	3.0	3.6	3.8	3.2	3.0	5.4
Myelocytes	0.2	3.2	0	0	0	0.6	4.2	4.6	6.8	4.6	3.0	5.2
Metamyelocytes	0.2	2.6	0.2	0.4	0	0.3	3.2	3.5	4.0	4.8	4.2	5.4
Polymorphonuclears, young	2.2	9.0	1.4	0.4	4.0	3.3	10.0	13.6	14.2	11.4	11.0	13.4
Polymorphonuclears, mature	19.0	13.0	2.8	1.6	3.6	3.3	10.8	10.0	6.6	6.8	15.0	9.8
Eosinophiles	0.4	0.8	0	0.4	0	0	6.2	5.6	1.8	1.0	1.6	1.8
Basophiles	0.4	0.6	0.2	0	0	0	0.4	0.3	0.2	0.2	0.6	0.2
Plasma cells	0.2	0.4	0	0	0.3	0	0.6	0.6	2.2	6.0	1.0	1.2
Disintegrated	12.8	10.4	19.4	19.0	7.6	12.6	15.2	3.3	14.0	13.2	10.0	8.6
Unclassified	0	0	0	0	0	0	1.0	1.0	2.2	2.0	1.6	1.2
Lymphocytes	45.0	41.0	70.0	71.6	83.3	78.0	34.0	36.0	38.0	32.0	19.4	16.0
Lymphocytes, immature	8.6	3.0	5.6	5.0	0.3	0.6	5.0	2.6	2.5	2.0	14.0	11.0
Monocytes	11.4	4.6	0.2	0.2	0.3	0.3	2.0	2.1	1.0	1.2	2.6	1.6
Monocytes, immature	0	0	0	0	0	0	0	0	0	0	0	0
Megaloblasts	0	0	0	0	0	0	0	0	0	0	0	0
Erythroblasts	0	4.0	0.2	0.6	0	0	2.0	4.0	2.0	3.6	3.6	5.8
Normoblasts	0.6	6.0	1.0	0.8	0	0.6	3.6	10.6	5.2	7.0	10.8	11.8
Nucleated cells per cu. mm. (in thousands)	27	64	121	267	196	200	26	75	58	95		126

* *SP—Spinous process marrow.*

** *ST—Sternal marrow.*

TABLE III

DIFFERENTIAL CELL COUNTS OF THE STERNAL AND SPINOUS PROCESS MARROW OF PATIENTS WITH MYELOCYTIC LEUKEMIA, SECONDARY ANEMIA, AND PERNICIOUS ANEMIA

Cell type	Myelocytic leukemia				Secondary anemia								Pernicious anemia	
	Case No.													
	1		2		1		2		3		4		1	
	Source													
	SP*	ST**	SP	ST	SP	ST	SP	ST	SP	ST	SP	ST	SP	ST
Blasts	1.4	1.6	2.6	2.4	0.6	0.8	0.8	0.8	0.6	1.0	1.6	0.8	1.8	2.2
Promyelocytes	12.0	14.8	3.4	3.4	3.6	6.8	2.2	6.8	3.4	5.0	7.2	3.0	6.8	6.6
Myelocytes	11.8	13.8	3.8	3.0	5.6	8.2	6.6	7.2	2.6	5.5	8.0	7.4	6.0	4.0
Metamyelocytes	10.4	8.0	11.4	9.4	2.8	4.8	4.4	4.0	4.0	6.6	10.6	9.2	7.0	4.2
Polymorphonuclears, young	26.6	28.4	35.6	37.4	17.8	17.0	22.8	22.0	17.6	23.2	23.0	19.0	12.0	11.2
Polymorphonuclears, mature	20.2	19.0	21.2	23.4	21.8	13.2	18.2	19.8	29.2	14.2	18.6	24.8	7.0	8.8
Eosinophiles	2.4	2.0	2.8	3.8	3.2	4.0	2.6	3.2	2.4	6.0	0.8	0	2.6	2.8
Basophiles	3.6	3.2	10.0	12.0	0.4	0.2	0.4	0.4	0.2	0.2	0.4	0	0	0
Plasma cells	0	0	0	0.2	0.4	0.6	0.2	0.2	0.6	1.2	0.4	0	0	0.4
Disintegrated	4.0	4.0	3.0	1.8	15.6	12.0	8.8	9.8	7.4	9.5	5.8	5.0	14.0	13.0
Unclassified	1.0	0	0	0	0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	0	0
Lymphocytes	3.8	3.4	0.8	1.2	8.0	6.8	10.0	9.6	15.8	11.0	6.4	7.0	8.2	7.6
Lymphocytes, immature	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Monocytes	1.8	1.2	0.4	0.4	5.2	2.6	2.2	1.0	3.6	3.2	2.0	1.4	0	1.0
Monocytes, immature	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Megaloblasts	0	0	0	0	0	0.4	0	0	0	0.2	0.4	0.2	10.0	12.4
Erythroblasts	0.6	0.2	1.8	0.4	4.6	9.6	10.0	4.6	6.2	5.7	5.2	12.0	19.0	21.5
Normoblasts	1.2	0.6	3.2	1.2	10.4	4.0	10.8	11.6	6.4	7.0	10.0	11.4	5.6	5.0
Nucleated cells per cu. mm. (in thousands)			47	40	7	30	60	81	180	42	70	64		

* *SP—Spinous process marrow.*

** *ST—Sternal marrow.*

Discussion and Conclusions

The findings in the two groups of normal subjects support the conclusions of Huss *et al.* (1) and Loge (2), that there is a close similarity between marrow from the spinous process and from the sternum.

The results in the 13 abnormal cases show that this similarity also holds for various pathological conditions of the marrow. This suggests that the information which has accumulated concerning sternal marrow variations in disease should be equally applicable to spinous process marrow.

Since spinous process puncture is as easy, as safe, and gives the same information as sternal puncture while causing less discomfort to the patient, it is suggested that this is the procedure of choice.

Acknowledgments

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THE EFFECT OF NIACIN ON THE PYRIDINE NUCLEOTIDE CONTENT OF HUMAN BLOOD CELLS IN ANEMIA[1]

By M. C. Blanchaer, D. E. Bergsagel, Pamela Weiss, and Dorothy E. Jefferson

Abstract

The pyridine nucleotide content of the blood cells (PN/BC) of four normal individuals and five anemic patients was measured repeatedly before and after supplementing the diet with niacin. Daily doses of 50 mgm. nicotinic acid or nicotinamide failed to affect the values in the normal subjects and two well-nourished anemic patients. The same dose of nicotinic acid rapidly raised the PN/BC of three poorly nourished anemic patients to values approximating those of well-nourished subjects with similar degrees of anemia. The results obtained after saturation with niacin confirmed the previous report that the PN/BC is higher in anemic patients than in normal controls and that a negative correlation exists between the pyridine nucleotide values and the severity of the anemia. In spite of continued niacin therapy, correction of the anemia was accompanied by a gradual decrease in the values until they approached those of the normal subjects. The present findings also confirm the earlier report that changes in the PN/BC are apparently independent of moderate variations in the number of circulating leucocytes and reticulocytes and bear no relationship to the size or hemoglobin content of the red cells. The significance of these findings in relation to human niacin nutrition is discussed.

Introduction

In a recent study (2) it was shown that the pyridine nucleotide content of the blood cells (PN/BC) of well-nourished but anemic patients is higher than that of normal subjects. A negative correlation was observed between the PN/BC and the severity of the anemia when the latter was expressed as the logarithm of either the red cell count or the hemoglobin concentration. Since the PN/BC of poorly nourished patients was distinctly lower than that of well nourished subjects with a similar degree of anemia, it was felt that such measurements might prove of value in the assessment of human nutrition.

The present study deals with the effect of niacin therapy on the PN/BC of normal subjects and anemic patients in various states of nutritional sufficiency. The influence of changes in the red cell count, hemoglobin concentration, and leucocyte count on the PN/BC was also investigated.

Methods

The subjects were four apparently healthy laboratory workers and five patients suffering from various types of primary and secondary anemia. Clinical data pertaining to the nutritional status of each patient may be found in the section dealing with the results.

[1] *Manuscript received February 24, 1950.*

Contribution from the Nutrition Laboratory of the Department of Physiology and Medical Research, The University of Manitoba, and from The Winnipeg General Hospital, Winnipeg, Canada.

The patients were investigated in the following manner. Hematologic and PN/BC measurements were made before and after supplementing an adequate diet, offered *ad libitum*, with niacin. The daily niacin supplement in most cases consisted of 50 mgm. nicotinamide or nicotinic acid. One patient received instead 10 mgm. nicotinamide daily in the form of a multiple vitamin capsule ("Supplavite"). Depending on clinical expediency, specific treatment of the anemia was begun before or after the patients had been placed on the increased level of niacin intake.

A similar plan was adopted in the investigation of the normal laboratory workers, except that these subjects were maintained for longer periods on the various levels of niacin intake.

Details of the hematological and chemical methods used in this work have been described (2). The fluorometric procedure (6) used in estimating the PN/BC does not distinguish between diphosphopyridine nucleotide and triphosphopyridine nucleotide. The blood cell PN content calculated as diphosphopyridine nucleotide was expressed in micrograms per milliliter of cells (PN/ml.), micrograms per gram of hemoglobin (PN/Hb), and micrograms per billion (10^9) red cells (PN/RBC).

Results

The effect of niacin therapy on the PN/BC of four apparently healthy laboratory workers is shown in Table I. Supplementing the diet of these subjects with a vitamin capsule or 50 mgm. nicotinic acid or nicotinamide daily resulted in minor fluctuations in the cellular PN content. These changes were similar in magnitude to the spontaneous variations reported by previous workers (5). The values at no time approached the high PN/BC found in well-nourished anemic patients (2). Although no comparable data is available in the literature on the effect of niacin in physiological amounts on the PN/BC, it is of interest that large doses of nicotinic acid (20 mgm. per kilo per day) produce a rapid elevation of the PN/BC (1, 4) while similar doses of nicotinamide have no effect (3).

The effect of adding 10 mgm. nicotinamide daily to the diet of a well-nourished woman of 35 years with a primary iron deficiency anemia is shown in Fig. 1A. During the first 17 days of the investigation, a decrease in the red cell count and hemoglobin concentration occurred which was accompanied by a small rise in the PN/RBC and PN/Hb values. Iron therapy produced a prompt improvement in the anemia. However, in spite of continued niacin therapy, the PN/BC decreased as the hemoglobin concentration and red cell count rose. The changes in the PN levels appeared to be unrelated to variations in the leucocyte count.

The second patient studied was a woman of 75 years with severe pernicious anemia. No neurological or gastrointestinal disturbances were present. No weight loss had occurred although there was a history of recent anorexia. The effect of nicotinic acid and parenteral liver extract therapy on the blood cell PN levels of this subject is shown in Fig. 1B. Administration of nicotinic

TABLE I

THE EFFECT OF NIACIN THERAPY ON THE PYRIDINE NUCLEOTIDE CONTENT OF BLOOD CELLS OF NORMAL SUBJECTS

Subject	Day	W.B.C. per cu.mm.	R.B.C. millions per cu.mm.	Hemoglobin, gm.%	Hematocrit	M.C.V.* μμl.	M.C.H.C.** %	PN/BC			Niacin therapy, mgm. per day
								PN/ml.***	PN/RBC†	PN/Hb††	
1	0	7400	6.4	16.4	48.5	78	34	72	5.5	210	Basal
	21	—	5.7	15.8	47.5	82	33	73	6.0	220	Nicotinamide, 10 mgm., 20 days
	36	4560	5.0	15.2	48.2	94	32	75	7.3	240	Nicotinic acid, 50 mgm., 14 days
	55	—	—	15.4	47.4	—	—	60	—	180	Nicotinamide, 50 mgm., 18 days
	160	8280	5.1	15.9	51.5	101	31	66	6.7	210	Basal for 104 days
2	0	3600	4.9	13.4	42.6	86	32	65	5.6	210	Basal
	18	—	4.5	13.2	41.9	92	32	60	5.6	190	Nicotinamide, 10 mgm., 16 days
	43	—	—	14.7	44.5	—	—	57	—	170	Nicotinic acid, 50 mgm., 14 days
3	0	5500	5.3	14.8	50.5	96	28	64	6.1	220	Basal
	14	7100	6.1	15.5	50.5	83	31	74	6.1	240	Nicotinic acid, 50 mgm., 14 days
4	0	5600	4.2	13.8	45.5	108	30	58	6.2	190	Basal
	20	8550	6.0	14.4	48.0	81	30	70	5.7	240	Nicotinic acid, 50 mgm., 19 days
	41	9225	6.7	15.5	49.5	74	31	64	4.7	200	Nicotinamide, 50 mgm., 19 days

* *Mean corpuscular volume (red cells).*
** *Mean corpuscular hemoglobin concentration.*
*** *Micrograms pyridine nucleotide per milliliter blood cells.*
† *Micrograms pyridine nucleotide per 10^9 red cells.*
†† *Micrograms pyridine nucleotide per gram hemoglobin.*

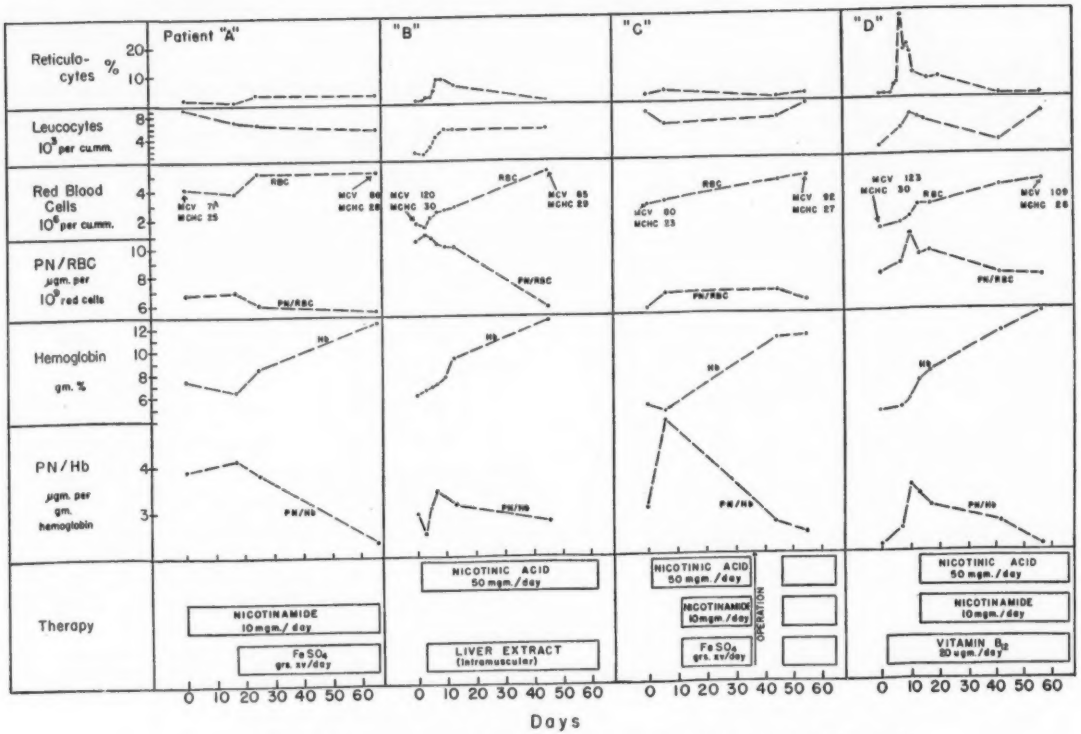

FIG. 1. *Effect of therapy on the blood cells and their pyridine nucleotide content in anemia.*

acid on the first and second day of the study resulted in a small increase in the PN/RBC and a decrease in the PN/Hb values. Intramuscular injections of liver extract produced a moderate reticulocyte response and a rapid rise in the red cell count, hemoglobin concentration, and leucocyte count. It will be seen that the appearance of increasing numbers of reticulocytes beginning on the fourth day was accompanied by a fall in the PN/RBC values. This observation eliminated the possibility that the simultaneous rise in the PN/Hb was entirely due to the increased number of circulating immature and young red cells and casts doubt on the suggestion of Kohn and Bernheim (5) that the elevated PN/BC of a case of pernicious anemia in their series was due to the presence of reticulocytes. The changes in the PN/BC were not well correlated with the characteristic response of the leucocyte count in pernicious anemia to liver therapy. In spite of a continued elevated nicotinic acid intake, the PN/Hb and PN/RBC values decreased as the hemoglobin concentration and red cell count rose until the cellular PN content approached that of the normal subjects in Table I.

The remaining three patients studied were poorly nourished. The first of these was a man of 59 years with a severe microcytic hypochromic anemia due to chronic bleeding. This subject habitually consumed an inadequate diet and reported recent anorexia and weight loss. No overt physical signs of vitamin deficiency were present. The loss of blood and the subject's poor nutritional status was traced to a partial obstruction of the stoma of a gastrojejunostomy by a phytobezoar. The effect on the PN/BC of nicotinic acid and iron therapy followed by operative removal of the gastric obstruction is shown in Fig. 1C. Administration of nicotinic acid alone resulted in a marked rise in the PN/RBC and PN/Hb values by the seventh day of the study. Eleven days postoperatively, the red cell count had risen to 4.3 millions per cu.mm. and the hemoglobin concentration to 10.9 gm.%. The total plasma protein concentration rose during the course of treatment from 4.6 to 7.9 gm.%. This improvement in the anemia, as in the previous patients, was associated with a drop in the blood cell PN content. It seems unlikely that the decrease in the cellular PN levels was due to a deleterious effect of the operation on the subject's nutritional status since resumption of intensive niacin therapy produced no rise in the PN values. No correlation was observed between the changes in the blood cell PN content and the leucocyte count.

The fourth patient investigated was a man of 65 years with scurvy, massive edema, steatorrhoea, and a severe macrocytic anemia associated with nontropical sprue. On admission to hospital the plasma protein concentration was 4.1 gm.%. No physical signs of niacin deficiency were found. The data on this subject are shown in Fig. 1D. Parenteral vitamin B_{12} therapy produced a rapid rise in the number of circulating leucocytes and reticulocytes which coincided with a moderate increase in the PN/BC. Although the latter rose still further on niacin therapy, the PN values subsequently decreased as the hemoglobin concentration and red cell count increased.

TABLE II

THE EFFECT OF NIACIN THERAPY ON THE PYRIDINE NUCLEOTIDE CONTENT OF THE BLOOD CELLS IN A CASE OF PERNICIOUS ANEMIA

Patient E

Day	W.B.C. per cu.mm.	R.B.C. millions per cu.mm.	Hemoglobin, gm.%	Hematocrit	Reticulocytes, %	M.C.V.* μμl.	M.C.H.C.** %	PN/BC			Niacin therapy, mgm. per day
								PN/ml.***	PN/RBC†	PN/Hb††	
0	3800	1.3	4.6	15.4	0.9	119	30	74	8.8	250	Basal
3	2700	1.2	4.8	14.1	0.7	117	34	106	12.3	310	Nicotinic acid, 50 mgm. 2 days
6	4800	1.0	4.4	14.3	0.4	143	31	102	14.5	330	Nicotinic acid, 50 mgm. 5 days

* *Mean corpuscular volume (red cells).*
** *Mean corpuscular hemoglobin concentration.*
*** *Micrograms pyridine nucleotide per milliliter blood cells.*
† *Micrograms pyridine nucleotide per 10^9 red cells.*
†† *Micrograms pyridine nucleotide per gram hemoglobin.*

Since these results suggested that the initial increase in the PN/BC of poorly nourished patients with macrocytic anemia is dependent upon a simultaneous rise in the leucocyte and reticulocyte counts, a woman of 65 years with untreated pernicious anemia was studied. The diet had been low in vegetables, fresh fruits, and animal protein for some time prior to investigation. The results are presented in Table II. Addition of 50 mgm. nicotinic acid daily to the diet was followed by a marked rise in the PN/Hb and PN/RBC values in the absence of any significant change in the red cell count, hemoglobin concentration, leucocyte and reticulocyte counts.

Previous work (2) has indicated that a negative correlation exists between the PN/Hb values of well nourished subjects and the logarithm of the hemoglobin concentration. It was also found that the PN/Hb levels of poorly

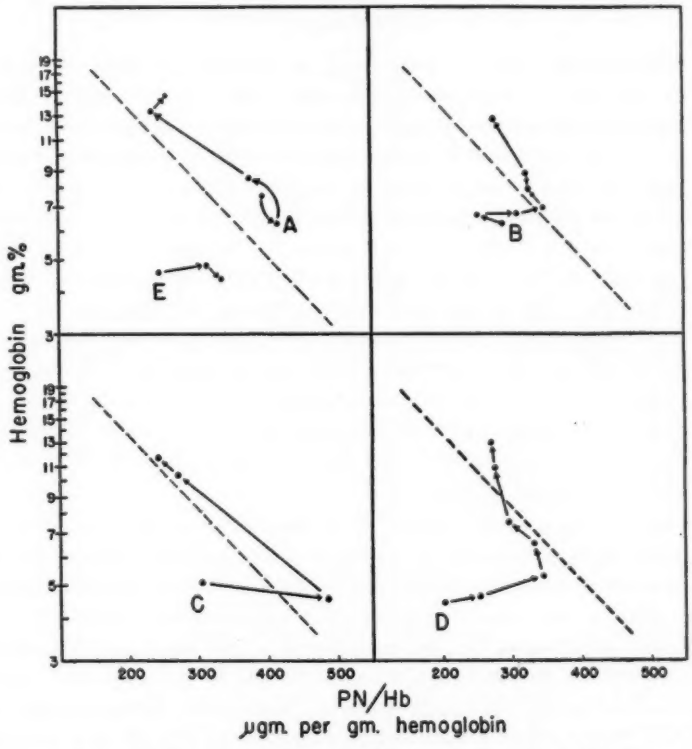

FIG. 2. *Data from Fig. 1 and Table II plotted to show the relationship of PN/Hb to hemoglobin concentration.*

nourished patients were lower than those of well nourished subjects with similar degrees of anemia. In order to re-examine this relationship, the corresponding data on the five patients in the present study were plotted in Fig. 2.

For purposes of comparison, the approximate regression line of the relationship previously found (2) between these variables in well-nourished subjects has been included in the figure.

It may be seen that the basal PN/Hb value of each patient, with the exception of the first, was lower than the mean value for well nourished subjects with comparable hemoglobin concentrations. In each case there was good agreement between the patient's general nutritional status and the distance of the initial PN/Hb value from the regression line. With the exception of the first patient, the PN/Hb values rose following niacin therapy and tended to approach the "normal" reference line. As the anemia responded to treatment, the PN levels decreased in spite of a continued elevated niacin intake.

Discussion

In presenting the results of this study, an attempt has been made to differentiate between the effect of supplementary niacin on the PN/BC and the changes which these values undergo during the response of anemias to specific therapy. It was found that in anemic patients with no evidence of malnutrition, supplementing the diet with niacin had little effect on the PN/BC, while in malnourished patients supplementation raised the values. Correction of the anemia after the values had been raised to "normal" was followed by a gradual decrease in the cellular PN levels until they approached those of the healthy controls. The latter observation confirms the negative correlation between the severity of the anemia and the PN/BC previously described (2) in a group of well nourished subjects with varying degrees of anemia. The present findings also confirm the earlier report that changes in the PN/BC are apparently independent of moderate variations in the number of circulating leucocytes and reticulocytes and that the changes occur in both macrocytic and microcytic types of anemia.

Since none of the patients referred to in this or the previous report showed overt clinical signs of pellagra, no evidence is available to indicate that malnourished subjects are deleteriously affected by low PN/BC levels. However, it is clear that the low values of patients with signs of general malnutrition can be raised by niacin therapy to the levels found in well nourished subjects with comparable degrees of anemia and that the PN/BC in well nourished subjects cannot be raised further by niacin therapy. While such findings suggest that low PN/BC values reflect a state of niacin deficiency, it is felt that this interpretation of the results is acceptable only if it can be shown that the lowered values are associated with a disturbed metabolic activity of the blood cells or can be correlated with some other sign of deficiency. Should further work provide such evidence, it appears that PN/BC values, taking into consideration the effect of anemia, will provide a sensitive index of niacin nutrition.

Acknowledgments

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ON RAT SERUM LIPASE

1. A SIMPLE MICROMETHOD FOR ESTIMATION OF LIPASE ACTIVITY IN RAT SERUM[1]

By Jules Tuba and Robert Hoare

Abstract

A titrimetric micromethod is described for estimating the lipolytic action of rat serum on tributyrin, tripropionin, and ethyl butyrate. A study has been made of factors affecting the action of the enzyme. Values for serum lipase levels of normal adult male and female rats are presented for the three substrates.

Introduction

A variety of names is given in the literature to the enzyme found in the serum of rats which is capable of hydrolyzing the lower molecular weight triglyceride fats and the fatty acid esters of monatomic alcohols. It is proposed to refer to the enzyme system which hydrolyzes tributyrin, tripropionin, and ethyl butyrate as lipase and its action as lipolysis.

The study of the lipolytic activity of rat serum under various experimental conditions necessitated the development of a suitable and simple microtechnique. In addition, it was essential to obtain the normal range of activity of the enzyme for each of the above substrates.

Experimental

Adult albino rats, Wistar strain, were used for the investigations reported here. They were fed Purina Fox Checkers and tap water ad libitum.

Blood for lipase determinations was always taken at 8.00 a.m. When the lipase activity of an individual animal was followed at various time intervals, sufficient tail blood was taken to yield at least 0.2 ml. serum, the amount required for a control and an experimental determination. Larger amounts of serum, required for studies of kinetics, were obtained by decapitation and pooling of the blood from several animals. Lipase concentrations were determined usually within 24 hr. after the serum was obtained, although the enzyme level has been found to be unchanged up to two weeks if stored at 5° C. without preservatives.

Factors Affecting the Action of Lipase

It was decided to avoid the use of emulsifying agents, such as bile or bile salts, in order to maintain as simple a procedure as possible. It was found that mechanical agitation of the enzyme–buffer–substrate mixture during the

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Contribution from the Department of Biochemistry, University of Alberta, Edmonton, Alta., with financial assistance from the National Research Council, Ottawa, Canada.

digestion period was sufficient to maintain the substrate in a state of fine dispersion, and the use of an emulsifier did not increase the amount of hydrolysis. Goldstein, Epstein, and Roe (2) report very satisfactory lipase estimations in a macromethod where they dispense with an emulsifying agent and use a hand homogenizer to emulsify the substrate and buffer. An important factor in the choice of a buffer is its ability to prevent a pH drift from the optimum due to acid production during the digestion period. Most satisfactory in this regard are sodium diethyl barbiturate – hydrochloric acid buffers, covering the pH range 6.8 to 8.6.

The influence of pH upon the activity of lipase for the three substrates used in the investigations is shown in Fig. 1. The pH optima are; 8.05 for tributyrin, 7.6 for tripropionin, and 7.2 for ethyl butyrate; and the respective decreases in pH during hydrolysis are 0.15, 0.35, and 0.25 units. The

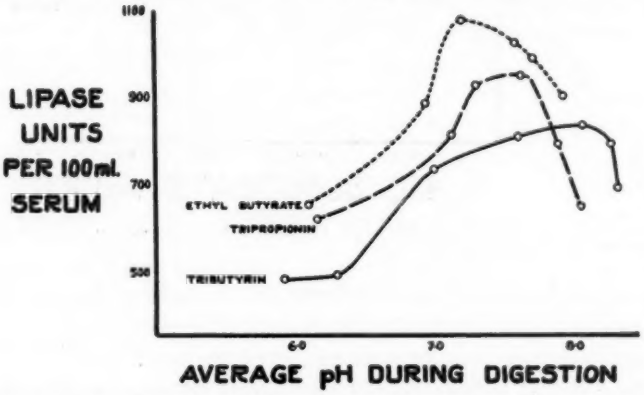

FIG. 1. *Effect of pH on hydrolysis of tributyrin, tripropionin, and ethyl butyrate by rat serum lipase.*

amount of the veronal buffer required in a digestion mixture varied with the substrate used: 1.0 ml. for tributyrin: 2.0 ml. for tripropionin: and 3.0 ml. for ethyl butyrate. (See procedure below.)

The time interval chosen for enzyme lipolysis was kept within the linear part of the time–activity curve. This minimized the pH drift away from the optimum. Accordingly the most suitable period for hydrolysis was found to be 30 min. for tributyrin, 15 min. for tripropionin, and 30 min. for ethyl butyrate. These intervals were long enough in each case to set free sufficient acid to be accurately estimated.

The relationship between concentration of substrate and enzyme activity is illustrated in Fig. 2 for each of the three substrates. It was decided to use 0.02 ml. tributyrin; 0.025 ml. tripropionin; and 0.015 ml. ethyl butyrate. (See the procedure below.)

After consideration of the several methods described by various workers for estimating the amount of fatty acid set free during lipolysis, a simple titrimetric method was adopted with sodium hydroxide, using phenolphthalein as an indicator.

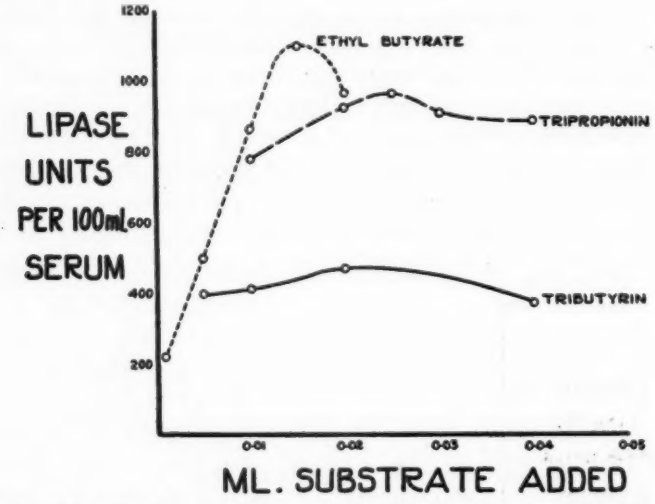

FIG. 2. *Effect of substrate concentration on hydrolysis of tributyrin, tripropionin, and ethyl butyrate by rat serum lipase.*

Procedure for Microdetermination of Serum Lipase Activity

Reagents

Substrates: tributyrin, tripropionin, ethyl butyrate.

Buffers: 0.1 M sodium diethyl barbiturate adjusted with hydrochloric acid to pH 8.2 for tributyrin, pH 7.8 for tripropionin, and pH 7.4 for ethyl butyrate.

Lipase inactivator: 95% ethyl alcohol.

Sodium hydroxide: 0.025 *N*.

The method for tributyrin will be described in detail and any necessary modifications for tripropionin (P) and ethyl butyrate (EB) will be indicated in brackets.

Micro-Kjeldahl tubes of approximately 5 ml. capacity are used for estimating enzyme activity. Into one of these tubes are pipetted 0.1 ml. serum, 0.2 ml. water, and 1.0 ml. buffer of pH 8.2 (P = 2.0 ml. buffer pH 7.8; EB = 3.0 ml. buffer pH 7.4). The mixture is warmed to 37° C., and then 0.020 ml. tributyrin (P = 0.025 ml.; EB = 0.015 ml.), previously warmed to 37° C., is added with rapid shaking. The dilution of serum in digestion mixtures containing tributyrin, tripropionin, and ethyl butyrate is 1 : 13, 1 : 23, and 1 : 33, respectively. While a constant total volume for the three substrates

would be advantageous, it was found that the above dilutions were most suitable to obtain optimum buffer action and to keep the digestion mixture at a minimum volume. The same procedure was used in establishing the relationships expressed in Figs. 1 and 2. The contents of the tube are mechanically agitated for 30 min. (P = 15 min.; EB = 30 min.) at 37° C. on a Warburg shaker at a rate of 120 swings a minute, which maintains the substrate in a finely dispersed state. For tributyrin, the pH of the contents of the experimental tube at the end of 30 min. is 7.9, the value reported in an earlier paper (8). However, the average of the initial and the terminal pH values was 8.05 in a number of experiments, and this is considered a better representation of the situation.

Lipase activity is terminated and the serum proteins are precipitated by the addition of 3 ml. 95% ethyl alcohol (P = 5 ml.; EB = 8 ml.). The mixture is centrifuged and the supernatant is poured into a 50 ml. Erlenmeyer flask, which is stoppered at once to minimize the absorption of carbon dioxide. The contents of the flask are titrated with 0.025 *N* sodium hydroxide, using phenolphthalein as an indicator, to a faint but persistent pink color.

A control tube is used, which is identical with the experimental tube except that the serum is boiled before buffer and substrate are added.

Enzyme activity is measured by the difference between the titration values of the experimental and control tubes. The lipase activity of serum in units is equivalent to the number of ml. of 0.025 *N* sodium hydroxide required to neutralize the amount of fatty acid set free by the enzyme contained in 100 ml. serum under the above conditions. One ml. of 0.025 *N* base is equivalent to one lipase unit. Because conditions of hydrolysis are different for each substrate, in all experiments the nature of the substance undergoing hydrolysis should be indicated.

Influence of Enzyme Concentration on Reaction Velocity

The effect of concentration of the enzyme and its activity with the three substrates is shown in Fig. 3. Varying concentrations of serum are used, while all other substances in the digestion mixtures are present in the quantities indicated above under procedure for microdetermination.

It is apparent from Fig. 3 that the enzyme contained in 0.1 ml. serum (the volume used in routine determinations) is saturated by the optimal concentrations of substrate shown in Fig. 2. The straight line relationship is maintained for at least 50% greater concentration than normal levels of the enzyme in rat sera.

Routine determinations of the lipase activity of serum can be determined most satisfactorily with tributyrin as a substrate. The change from optimum pH during digestion is less than with the other two substrates. The total volume of the digestion mixture plus alcohol, used to terminate enzyme action on this substrate, is a convenient one to handle. The relationship between enzyme concentration and activity permits the saturation by tributyrin of levels of enzyme well above the normal range in rat serum.

Replicates on the same serum, using this relatively simple and rapid micro-method, have repeatedly shown agreement well within the limits of 5% experimental error. The amount of substrate hydrolyzed by normal adult

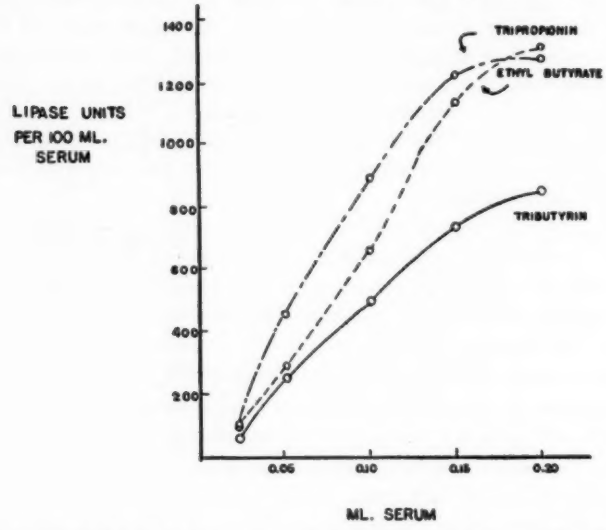

FIG. 3. *Relationship between enzyme concentration and lipase activity for tributyrin, tripropionin, and ethyl butyrate.*

rat serum lipase is approximately 6% for tributyrin, 7% for tripropionin, and 12% for ethyl butyrate under the above experimental conditions.

Activation and Inhibition

The accelerating or retarding effect of a limited number of substances on the activity of serum lipase was investigated. Solutions of these substances were pipetted into the experimental tubes in place of the 0.2 ml. water which is usually added, and they were incubated with the serum–buffer mixture for 15 min. before the addition of tributyrin.

The activation of lipase indicated in Table I by calcium chloride is in conformity with the findings of Kraut, Weischer, and Hügel (3), who noted an increase in hydrolysis of pancreatic lipase at pH 8.9 when calcium chloride was added. The same authors report an activation by sodium glycocholate. We were unable to obtain an enhancement of rat serum lipase activity with sodium taurocholate (not included in table), which is in agreement with the findings of Parfentjev, Devrient, and Sokoloff (6) for rabbit serum lipase. An appreciable inhibition was produced with eserine, using a concentration somewhat greater than that of 10^{-5} *M* which Mendel, Mundell, and Rudney (4) have shown to be sufficient to inhibit completely the cholinesterases of guinea pig plasma. A very marked decrease in lipolysis followed the addition of hexaethyl tetraphosphate. DuBois and Mangun (1) obtained 60%

TABLE I

EFFECT OF VARIOUS SUBSTANCES ON THE ACTIVITY OF RAT SERUM LIPASE

Substance	Lipase units/100 ml. serum		Effect on lipase activity
	Control	Experimental	
Calcium chloride 3.4×10^{-2} *M*	685	850	+24%
Eserine sulphate 2.3×10^{-4} *M*	470	290	−38%
Hexaethyl tetraphosphate 1.9×10^{-3} *M*	620	85	−86%
Sodium fluoride 7.4×10^{-3} *M*	415	40	−91%

inhibition of rat serum cholinesterase using this same inhibitor at a concentration of 10^{-7} *M*. Almost complete cessation of activity of the enzyme was produced by sodium fluoride. This is an indication, according to Singer (7), that serum lipase is a calcium-requiring enzyme. Evidence in favor of this was found in an additional experiment. The presence of 7.4×10^{-2} *M* sodium fluoride in an experimental tube lowered the activity of the enzyme 75% (from 620 to 150 units lipase per 100 ml.). In another tube the enzyme was incubated for 30 min. with 7.4×10^{-2} *M* sodium fluoride, and then 3.4×10^{-2} *M* calcium chloride was added just before the substrate. The enzyme activity in this case was 705 units per 100 ml. or about 13.5% above the control value.

Normal Serum Lipase Values for Adult Rats

These are presented in Table II for the three substrates, tributyrin, tripropionin, and ethyl butyrate. The numbers of animals for the latter two substrates are not large, but they are sufficient to indicate that the rate of

TABLE II

THE MEAN, THE RANGE, AND THE STANDARD DEVIATION FOR SERUM LIPASE OF NORMAL ADULT RATS FOR TRIBUTYRIN, TRIPROPIONIN, AND ETHYL BUTYRATE

Number of animals	Sex	Substrate	Mean	Range	Standard deviation
56	Male	Tributyrin	596	420-780	±101
16	Female	Tributyrin	628	490-840	± 84
14	Male	Tripropionin	858	720-1050	±120
6	Female	Tripropionin	735	630-950	±104
16	Male	Ethyl butyrate	734	590-900	± 84
6	Female	Ethyl butyrate	737	630-900	± 93

hydrolysis of tripropionin (in 15 min. digestion period) is much greater than for tributyrin, or ethyl butyrate (both with 30 min. digestion periods). Mundell (5) observed that the plasma of mature female rats had greater activity toward acetylcholine than males, due mainly to the nonspecific

cholinesterase. It does not appear from the data of Table II that there is any significant difference in the ability of serum lipase of adult male and female rats to hydrolyze tributyrin.

The ratios of hydrolysis of ethyl butyrate and tributyrin differ in Fig. 2 and Table II. The large volumes of serum required to determine the results expressed in Fig. 2 were obtained by decapitating several animals and pooling their sera. Different pools were used for each substrate. The ratio of hydrolysis for these two substrates indicated in Table II (i.e. above 1.2) is based on sera obtained from tail blood as indicated above. In subsequent experiments on normal adult rats, the ratio has remained constant.

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SOME PROPERTIES OF NORMAL CHICK ALLANTOIC FLUID IN RELATION TO INFLUENZA VIRUS HEMAGGLUTINATION[1]

By J. R. Polley, M. M. Burr, and A. L. Gillen

Abstract

The pH, oxidation–reduction potential, total solids, nonspecific hemagglutination titer, and the titer of the inhibitor of influenza virus hemagglutination have been determined in the normal allantoic fluid of the chick embryo as the incubation time increased. These determinations were repeated after the allantoic fluid had been stored for 48 hr. at 0° C. It was found that as the incubation time increased (1) the pH decreased progressively, (2) the Eh remained positive throughout, (3) the total solid content increased, (4) the nonspecific hemagglutination titer remained negligible, (5) the inhibition titer increased rapidly. Storage for 48 hr. at 0° C. produced no change other than a small rise in the pH. By buffering the allantoic fluid *in vivo*, it appeared that the increasing inhibition titer was not directly related to the decreasing pH. The inhibition titer of the allantoic fluid was not decreased after dialysis, indicating that it is not affected by the amount of urates in the fluid. Thus it appears that the inhibition titer is related directly to a component of the increasing total solids which is unidentified as yet.

At the present time, the embryonated egg is in widespread use for the propagation and passage of numerous viruses. The isolation and identification of many human and animal viruses involves passage through the fertile chicken egg (1, 9), followed by various procedures for the isolation and purification of the viruses from the embryo fluids.

It has been shown that the growth of viruses and their stability are affected by the pH, oxidation–reduction potential, and the temperature of the medium (2, 5, 10, 12). Beard *et al.* (11) found that with mumps virus, the optimal range of pH stability was from 5.8 to 8.0. Influenza A virus (PR8) was most stable at about pH 7 while influenza B virus (Lee) was most stable at pH 7.9 or greater (4). Usually, a neutral or slightly alkaline pH, a reducing medium, and a temperature of about 35°-37° C. favor virus growth.

Changes in the pH and Eh values of the allantoic fluid of normal and influenza infected eggs have been reported (6). With normal allantoic fluid the Eh was usually positive and the pH decreased as the age of the embryo increased. In 1947, Svedmyr (8) showed that normal allantoic fluid contained an inhibitor of influenza virus hemagglutination. Hardy and Horsfall (3) noted that this inhibitor increased with the time of incubation of the eggs and that it was capable of combining with influenza A virus. Following this combination, there was only partial dissociation.

Since a given virus, such as influenza, has an optimal pH for growth and stability, it seems possible that a decreasing pH of allantoic fluid with increasing time of incubation would represent a detriment for continued multiplication. Also, since it has been reported that the titer of the inhibitor of virus hemagglutination increases with time, it seems possible that the low yields of

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Contribution from the Laboratory of Hygiene, Ottawa, Canada.

virus found beyond certain times of incubation might be due, at least in part, to the fact that the true virus titer was obscured by an increasing inhibitor content. Hence, the present study of normal allantoic fluid was undertaken to follow, not only any changes in the pH and Eh of the fluid but any simultaneous changes which might occur (1) in the titer of the inhibitor of influenza virus hemagglutination and (2) in nonspecific hemagglutination. By giving quantitative expression to these properties determined simultaneously, it was hoped that it might be possible to correlate them with known conditions for optimal virus growth and to demonstrate their effect on virus assays.

Experimental

White Leghorn eggs received from a single hatchery were used. On arrival, the eggs were weighed individually and only eggs weighing between 55-58 gm. were selected for immediate incubation. The eggs were incubated on their side at 37.5° ± 0.5° C. at a relative humidity of 80% in a forced-draught incubator.

The allantoic fluid was harvested under aseptic conditions from viable embryos without rupturing the amniotic sac or the blood vessels of the chorioallantoic membrane. The allantoic fluid from each egg was placed in a separate sterile test tube. The pH, nonspecific hemagglutination, and inhibitor determinations were made immediately on a portion of each specimen. The remaining fluid was placed in an icebox at 0° C. for 48 hr. and then the above determinations were repeated to observe any changes on storage.

The determinations of the pH and the oxidation–reduction potential, Eh, were made using a Beckman model G pH meter. For the pH measurements, micro glass and calomel electrodes supplied with the instrument were used. To determine the Eh, a No. 281 platinum electrode was substituted for the glass electrode in the circuit.

Aliquots of the allantoic fluid samples were tested for the presence of nonspecific hemagglutinins by Salk's method (7).

For the titration of inhibitors of influenza virus hemagglutination in the normal allantoic fluid samples, serial twofold dilutions of known PR8-infected allantoic fluid were prepared in saline. It is important to point out that with these tests to be described, the titer of inhibitor is influenced by the titer of the virus used to determine it (3), hence a common pool of PR8 influenza infected allantoic fluid was used throughout this study. To 0.4 ml. of each dilution of virus was added 0.1 ml. of the allantoic fluid sample under test. The tubes were shaken and 0.5 ml. of a 0.25% suspension in saline of washed chicken cells was added. The tubes were shaken again and kept at room temperature until the cells had settled completely. A control titration of the virus in saline was made at the same time. End points were taken as the last dilution showing complete hemagglutination by Salk's method (7). The results were recorded as the degree of inhibition which is expressed as

the ratio of the virus titer in saline to the titer in the presence of normal allantoic fluid. This is similar to a method used by Hardy and Horsfall (3).

Repeating these tests with heated virus, we were able to confirm the finding that higher inhibition titers were obtained than with unheated virus (3).

Thus, these two titrations give the inhibition value against both heated and unheated virus. Using the heated virus, higher inhibition titers are obtained and there is a wider spread in the values which is desirable for comparing the results for increasing time of incubation. The corresponding values obtained with unheated virus indicate the actual degree of interference to be expected in ordinary virus titrations.

To determine the total solid content of the allantoic fluid, 1 ml. aliquots of each sample were added to tared crucibles and weighed again. The crucibles were placed in an oven at 105° C. for 24 hr. and were then weighed again when cool.

Results

The results of this study are presented in tabular rather than graphic form to present both the range and the standard deviations of the values with increasing time of incubation. For example, if a graph were plotted between the time of incubation and the mean pH values shown in Table I, the conclusion might be drawn that the decreasing pH was directly related to the

TABLE I

pH OF NORMAL ALLANTOIC FLUID WITH TIME OF INCUBATION

Time of incubation, days	No. of observations	pH range	Mean pH	After 48 hr. at 0° C.
10	30	7.4 – 8.2	7.9 ± 0.2	8.3 ± 0.3
11	30	7.1 – 8.1	7.7 ± 0.3	8.0 ± 0.3
12	18	7.2 – 8.0	7.5 ± 0.3	8.0 ± 0.3
13	18	6.5 – 7.9	7.3 ± 0.3	7.9 ± 0.3
14	30	6.4 – 7.9	7.0 ± 0.5	7.6 ± 0.5
15	30	5.5 – 7.5	6.5 ± 0.5	6.9 ± 0.5
16	24	5.3 – 7.0	5.8 ± 0.5	6.4 ± 0.6
17	20	5.0 – 6.7	5.6 ± 0.4	6.2 ± 0.5
18	8	5.2 – 6.7	5.6 ± 0.4	6.1 ± 0.5

incubation time. Actually, however, the range of pH values encountered shows that, although the average pH decreases with time, it is impossible to correlate the pH directly with the time of incubation for each individual egg.

From Table I it can be seen that the mean pH decreased with the time of incubation. The decrease was almost linear from 10 to 14 days, then there was a sharper drop to 16 days followed by a levelling off. After being stored for 48 hr. at 0° C., the pH of the allantoic fluid increased by about 0.5.

It can be seen from Table II that the Eh of the samples remained positive throughout the course of this study. Storage at 0° C. had no significant effect on the values.

TABLE II

Eh OF NORMAL ALLANTOIC FLUID WITH TIME OF INCUBATION

Time of incubation, days	No. of observations	Eh range, mv.	Mean Eh	After 48 hr. at 0° C.
10	20	+ 290 to + 340	+ 310 ± 40	300 ± 50
11	20	+ 290 to + 325	+ 305 ± 40	280 ± 45
12	20	+ 140 to + 280	+ 210 ± 70	250 ± 30
13	20	+ 185 to + 280	+ 245 ± 55	220 ± 45
14	20	+ 265 to + 340	+ 290 ± 35	260 ± 40
15	18	+ 190 to + 300	+ 240 ± 60	260 ± 70
16	20	+ 315 to + 360	+ 330 ± 25	290 ± 60
17	18	+ 285 to + 350	+ 325 ± 40	280 ± 70
18	8	+ 300 to + 360	+ 340 ± 40	300 ± 45

In Table III is shown the weight increase of the embryo, the rate of growth, expressed as gm. per day and the total solid content of the allantoic fluid. There was a gradual increase in the total solid content of the normal allantoic fluid with increasing age of the embryo. The amount, however, was only of the order of from 1 to 1.5 gm. per 100 cc. An indication of a low protein content was given by the fact that the addition of 10% trichloroacetic acid to samples produced only an immediate slight turbidity.

It was found that the titer of nonspecific hemagglutination remained less than 10 units throughout the time of incubation. Also, it did not increase after the fluid had been stored at 0° C. for 48 hr.

From Table IV it can be seen that the titer of the inhibition of influenza hemagglutination increased rapidly with time of incubation. With the heated virus, the inhibition titers are larger than with unheated virus but the range of values is similar in each case, usually being about a fourfold one. For example, at 13 days it is 16 – 64 with heated virus compared with 2 – 8 with unheated virus. Storage at 0° C. for two days did not cause any significant change in the titer, either with heated or unheated virus. The titer of the virus pool used for these tests was 2560 and since the first dilution tube

TABLE III

EMBRYO WEIGHT, RATE OF GROWTH, AND TOTAL SOLID CONTENT OF NORMAL ALLANTOIC FLUID WITH INCUBATION TIME

Time of incubation, days	No. of observations	Mean weight, gm.	Growth increment, gm.	Total solids, mgm./gm.
10	30	2.1 ± 0.2	—	8.5 ± 0.5
11	30	2.8 ± 0.2	0.7	9.7 ± 0.5
12	18	4.1 ± 0.3	1.3	9.9 ± 0.5
13	18	5.9 ± 0.3	1.8	10.6 ± 0.4
14	36	7.9 ± 0.4	2.0	10.2 ± 0.5
15	30	11.0 ± 0.4	2.1	10.5 ± 0.6
16	30	13.4 ± 0.5	2.4	12.0 ± 0.7
17	20	16.2 ± 1.0	2.8	15.1 ± 0.8
18	8	20.3 ± 0.4	4.1	15.3 ± 0.7

TABLE IV

TITER OF INHIBITOR OF INFLUENZA VIRUS HEMAGGLUTINATION IN NORMAL ALLANTOIC FLUID WITH TIME OF INCUBATION

Time of incubation, days	No. of observations	Inhibition titer			
		Immediate		After 48 hr. at 0° C.	
		Heated virus	Unheated virus	Heated virus	Unheated virus
10	30	4 – 16	2 – 4	8 – 16	2 – 4
11	30	8 – 16	2 – 4	8 – 16	2 – 4
12	18	8 – 32	2 – 8	2 – 16	2 – 8
13	18	16 – 64	2 – 8	8 – 16	2 – 8
14	30	16 – 64	4 – 8	32 – 128	4 – 8
15	30	32 – 128	8 – 32	32 – 128	8 – 16
16	24	128 – 256	8 – 32	128 – 256	8 – 32
17	18	128 – 256	32 – 64	256	32 – 128
18	8	256	64 – 128	256	64 – 128

was 1 : 10, the maximum inhibition titer that could be obtained here was 256. Using heated virus, this was reached on the 16th day of incubation. The inhibition titer with unheated virus was only 8 – 32 at this time but was

rapidly increasing. For practical purposes, an inhibition titer of 8 with unheated virus (which is often reached by the 12th day, the time of routine harvesting of influenza virus) would mean that a virus hemagglutination titer of 2560 would appear as only $\frac{2560}{8} = 320$ in the presence of normal allantoic fluid.

The following observations, which have been made as the time of incubation (age of embryo) increased, are presented together in an attempt to correlate the data: (1) the pH of the allantoic fluid decreased, (2) the pH increased after storage for two days at 0° C., (3) the oxidation–reduction potential remained positive throughout, (4) the nonspecific hemagglutination titer remained less than 10 units throughout, (5) the total solid content of the allantoic fluid increased, (6) the titer of the inhibitor of influenza virus hemagglutination increased rapidly. Since the oxidation–reduction potential remained positive and without any significant variation and since the nonspecific hemagglutination showed no increase, it appears that the increasing inhibition titer may be related to the increasing total solids and/or to the decreasing pH. That it was not the decreased pH per se which caused the rise in inhibition titer was shown by repeating the inhibition titrations in isotonic phosphate buffer, pH 7.0, and obtaining the same values. The fact that the pH of the allantoic fluid rose about 0.5 after storage in the icebox, without significant change in the inhibition titer, suggests that the decreasing pH and the increasing inhibition titer are not directly related. However, this pH change is not sufficiently great to be conclusive.

In an attempt to determine whether there is a direct relationship between the decreasing pH during the time of incubation and the increasing inhibition titer, the allantoic fluid was buffered *in vivo*. The inhibition titers were determined at given time intervals.

In a preliminary experiment, 1.0 ml. of sterile isotonic phosphate buffer, pH 7.5, was injected into the allantoic sac of embryos on the 10th day of incubation. A similar amount of sterile isotonic saline was injected into the allantoic sac of a control group of 10 day embryos. Normal (noninjected) fluids were also tested to serve as normal controls. Allantoic fluids were withdrawn for test on the 14th and 16th day of incubation. It was found that the average pH of the three groups of fluids had fallen to about the same extent. That the buffer had some small effect, however, was indicated by the fact that the buffered allantoic fluids showed less deviation in their pH values from the average value. The inhibition titers were similar in the three groups.

The experiment was repeated as above except that this time 2.0 ml. of 0.1 *M* phosphate buffer, pH 7.8, was used instead of 1.0 ml. of isotonic phosphate. An equivalent amount of saline was injected into others to serve as treated controls. The results are shown in Table V.

From Table V it can be seen that the action of the buffer is more marked on the 16th than on the 14th day of incubation. On both the 14th and 16th days, the inhibition titers for the three groups lie in similar ranges. There

TABLE V

pH AND INHIBITION TITER IN NORMAL AND BUFFERED ALLANTOIC FLUID

Treatment	Time of incubation, days	No. of observations	pH	Inhibition titer	Total solids, mgm./gm.
None	14	30	7.0 ± 0.5	16 – 64	10.2
Saline	14	24	6.7 ± 0.6	16 – 64	10.8
Buffer	14	20	7.1 ± 0.5	32 – 64	11.1
None	16	24	5.8 ± 0.5	12 – 256	12.0
Saline	16	20	6.0 ± 0.4	128 – 256	12.5
Buffer	16	20	6.6 ± 0.4	128 – 256	12.8

are differences in the pH values, however. Thus, the pH of normal allantoic fluid fell from 7.0 on the 14th day to 5.8 on the 16th day of incubation, while the buffered fluid decreased only from 7.1 to 6.6. The inhibition titer, however, decreased to the same extent in both normal and buffered fluids. Hence, it appears that the decreasing pH during incubation is not directly related to the increasing titer of the inhibitor of influenza virus hemagglutination.

To determine whether the inhibition titer was associated with the presence of urates in the allantoic fluid, aliquots of a pool of normal allantoic fluid were dialyzed in cellophane bags against 0.85% saline at 0°, 25°, and 37° C. for 48 hr. As controls, aliquots of the allantoic fluid were kept in test tubes at these temperatures. At the end of the dialyzing period, the inhibition titer of all samples was determined. It was found that dialysis for 48 hr. did not alter the inhibition titer at any of the above temperatures. This suggests that the inhibition is not due to the presence of urates in the normal allantoic fluid and that the inhibitor is a compound of relatively large molecular weight.

Discussion and Summary

One of the most important properties of normal allantoic fluid is its inhibitory action on influenza virus hemagglutination. In the process of harvesting virus from pooled infected allantoic fluids, the inclusion of fluids having little or no virus content decreases the titer of the pool by considerably more than would an equivalent amount of saline. The fact that it has been found that the optimal time for harvesting influenza-infected allantoic fluid is from the 12th – 13th day of incubation may be a reflection of the fact that beyond this time the rapidly increasing inhibitor content masks the true virus content by depressing the experimental titration value.

The increasing concentration of inhibitor in the allantoic fluid was associated with a decreasing pH and an increasing total solid content. By buffering

some allantoic fluid *in vivo* it was shown that the inhibition was not related directly to the decreasing pH. The inhibitory action of allantoic fluid was unchanged after dialysis, indicating that it was not due to the urate fraction of the total solids. Apparently, the increasing inhibition titer is related directly to an increasing component of relatively large molecular weight in the total solids which is unidentified as yet. Further studies are in progress.

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THE UPTAKE OF RADIOACTIVE PHOSPHORUS BY INFLUENZA VIRUS A (PR8 STRAIN)[1]

By A. F. Graham and Laurella McClelland

Abstract

Radioactive inorganic phosphorus placed in the allantoic sacs of embryonated eggs three hours after inoculation with influenza virus was incorporated into the structure of the virus during its growth. There was little or no direct exchange between the virus and radioactive inorganic phosphorus. The specific activity of purified labelled virus rose linearly with increasing amounts of radioactive phosphorus administered to the eggs. When radioactive phosphorus was placed in the allantoic sac 48 hr. before inoculation with influenza virus the newly formed labelled virus had a specific activity about 20% higher than when isotope was administered at the same time as virus. As the amount of isotope injected into each infected egg was increased up to 775 μrd. an increasing number of embryos died during the subsequent period of virus growth. The yield of virus from the surviving eggs was not less than from eggs which had not received radioactive phosphorus. Under the experimental conditions described the amount of isotope which could be introduced into influenza virus was not sufficient to permit the use of the marked virus in metabolism experiments in animals or embryonated eggs.

Introduction

The purpose of this work was to determine whether the elementary bodies of influenza virus could be labelled with radioactive phosphorus during their growth in the allantoic membrane of the embryonated egg. If purified influenza virus could be obtained with a sufficiently high content of isotope it was considered that a number of problems in the field of animal viruses would be open to new methods of study.

Thus, in the first place, it would be of interest to determine the fate of the isotope when mouse lung or allantoic membrane was infected with radioactive virus since this might throw some light on the mechanism of cell infection by the virus. Secondly, chemical analysis of radioactive virus would show whether the isotope was concentrated in one or more of the virus constituents, indicating that these constituents played a special role in virus growth. Thirdly, it would be of great interest to compare the rate of uptake of isotope by some of the phosphorus containing constituents of the normal and infected cell; this would give an indication as to whether virus infection alters the phosphorus metabolism of the cell. Fourthly, radioactive influenza virus might be useful in immunological studies along lines suggested by the work of Libby and Madison with labelled tobacco mosaic virus (9).

Some of these problems are general to the study of all viruses, and it appeared that a suitable model system for preliminary study would be that of influenza virus growing in the allantoic membrane. Conditions for growth, methods of purification, and chemical analysis of this virus have already been worked out in detail by previous investigators. Although no published work was available on the application of isotope techniques to the study of animal

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Contribution from Connaught Medical Research Laboratories, University of Toronto, Ont.

viruses, Cohen (1, 2) and Putnam and Kozloff (11) have investigated the growth of T_2, T_4, and T_6 bacteriophages on *Escherichia coli* in the presence of radioactive phosphorus. Since these bacterial viruses readily incorporated the isotope into their structures there was a strong suggestion that influenza virus would also become labelled during its growth in the fertile egg.

The present paper describes the conditions under which radioactive phosphorus was incorporated into the structure of influenza virus. A preliminary report of this work has already appeared (5).

Methods

Determination of Total Phosphorus

Total phosphorus was determined by the method of Kuttner and Lichenstein (8) with several modifications introduced to suit the present purpose.

An aliquot of the solution to be estimated was pipetted into a test tube graduated at the 15 ml. mark, and 1.0 ml. of 15 *N* sulphuric acid was added. The mixture was heated on an open flame until most of the water had been removed and digestion was continued on a sand bath over an electric hot plate for one hour. One drop of concentrated nitric acid was then added and the heating continued a further half hour. One to two ml. of distilled water was added and then evaporated off rapidly over an open flame. This procedure removed decomposition products of nitric acid which interfered seriously with the subsequent color development.

After addition of 2.0 ml. of 5.51% ammonium molybdate (w/v) the volume was made up to about 13 ml. with water and thoroughly mixed. To this was added 1.5 ml. stannous chloride (working solution prepared daily by diluting 1.0 ml. of stock solution, 10 gm. stannous chloride in 25 ml. concentrated hydrochloric acid, to 200 ml. with water) the tube being shaken continuously during addition. The volume was made up to 15 ml., mixed, allowed to stand 10 min. at room temperature, and the per cent transmittance read against distilled water at 630 mμ in a Coleman Spectrophotometer. The calibration curve was linear between 0 to 20 μgm. P.

Determination of Radioactive Phosphorus (P^{32})

Practically all the determinations of radioactivity were made on solutions of material in water, biological fluids, or organic solvents. The method used will be fully described (4).

Throughout this paper the activities of P^{32} sources are expressed as counts per minute (c.p.m.), that is, the number of impulses registered per minute on a Geiger-Müller counter. Radioactivity measurements were carried out in triplicate for each solution. Each source was measured with a standard deviation of 2 to 5% of the net counting rate (7) unless the net counting rate was less than about 20% of the background, when a standard deviation of 25% was considered to be sufficient accuracy. Since the method was calibrated (4) against a standard RaD + E source from the National Bureau of

Standards, the results may be converted to microrutherfords (μrd.) using the factor 1000 c.p.m. are equivalent to a β-ray activity of 85.8 μrd.

The radioactive phosphorus was obtained at monthly intervals from the National Research Council, Chalk River, as phosphoric acid in 0.005 *M* hydrochloric acid, carrier free. The decay rate of the material was checked experimentally from time to time, but, in general, corrections for decay of P^{32} during an experiment were calculated from a decay curve assuming a half-life of 14.5 days.

Determination of Protein Nitrogen

Protein nitrogen was estimated after digestion with sulphuric acid and Perhydrol by the Nessler method using the reagent described by Vanselow (16). The density of the developed brown color was determined at 460 mμ against the reagent blank. Ammonium sulphate was used as a standard, the calibration curve being linear from 0-60 μgm. N.

Determination of Infectivity of Influenza Virus

Serial tenfold dilutions of virus suspension in broth were injected in 0.2 ml. amounts into 11 day embryonated eggs by the allantoic route. Generally, at least six groups of seven eggs each were used for an infectivity titration. After incubation at 36° C. for 48 hr. a small quantity of allantoic fluid was aspirated from each egg and tested for haemagglutination with a washed suspension of chicken erythrocytes. The 50% infectivity end-point (ID_{50}) was calculated according to the method of Reed and Muench (12).

In many cases the virus content of solutions was estimated by the chicken red cell agglutination (CCA) test of Miller and Stanley (10). This test was used only as a rough indication of the amount of virus in a suspension, its reproducibility and accuracy being much inferior to the infectivity titration.

Experimental

Growth, Purification, and Properties of Influenza Virus

During the course of the work several methods for harvesting and purifying influenza virus were tried but the best preparations were obtained by the following procedure which is similar in many respects to that worked out by Taylor *et al.* (15).

Eleven-day embryonated eggs were inoculated by the allantoic route with 10-100 ID_{50} of influenza virus A (PR8 strain) in 0.2 ml. amount. After incubating the eggs for 48 hr. at 36° C. they were opened under sterile conditions and a large blood vessel of the inner chorioallantoic membrane was severed. The mixture of blood and allantoic fluid was aspirated into 250 ml. centrifuge bottles and allowed to stand at 5° C. for 18 hr. to permit complete agglutination of the red blood cells by the virus. Following centrifugation the agglutinated cells were suspended in ice-cold 0.85% (w/v) sodium chloride adjusted to pH 7.0, hereafter referred to as "saline", and again centrifuged. A volume of saline equivalent to 1/10 the original volume of allantoic fluid was then added and the virus eluted from the cells by incubating at 37° C.

for three hours. The cells were removed by sedimentation in a horizontal centrifuge at 1000 r.p.m. and the supernatant subjected to further centrifugation for 10 min. in an angle centrifuge at 2000 g. The supernatant solution thus obtained was centrifuged at 20,000 g for one hour at 5° C. in a Sorvall angle centrifuge to sediment the virus. A few drops of saline were added to the pellet after the supernatant solution was poured off. Whenever possible the mixture was allowed to stand overnight at 5° C. since the virus resuspended more readily under these conditions. The virus was then resuspended by pipetting for several minutes through a fine-tipped pipette, the volume made up to 1/100 the volume of original allantoic fluid with saline and centrifuged at 5° C. for 10 min. at 2000 g in an angle centrifuge to remove large particles. A second cycle of high and low speed centrifugation was performed on the supernatant solution.

The final virus suspension was a white opalescent liquid estimated by infectivity test to contain 55-70% of the virus in the original allantoic fluid. Such suspensions contained about 2.5 mgm. of virus per ml., calculated from phosphorus analyses, assuming that the virus contained 0.97% P (14).

In some cases the above procedure was modified in that the infected eggs were chilled at 5° C. for 18 hr. before harvesting, and washed chicken erythrocytes were added to the clear allantoic fluid to make a 2% suspension. This method was not as rapid as the one outlined above and suffered the further disadvantage that it was more difficult to maintain sterile conditions. Various media, such as the Ringer – calcium chloride solution described by Taylor *et al.* (15), and phosphate buffer, were used to resuspend the sedimented virus but offered no advantage over 0.85% sodium chloride solution.

Mounts were prepared from several of the purified virus suspensions, fixed in osmic acid vapor, and photographed in the electron microscope before and after shadow casting with chromium.* In every case the great majority of the particles observed were typical in size and shape of influenza virus with little other electron absorbing material present. In some cases a few of the filamentous forms often observed previously in preparations of this virus were apparent, see for example (13).

Freshly purified suspensions of virus gave a single boundary in the ultracentrifuge, Fig. 1, with a sedimentation constant of about 670×10^{-13} (uncorrected) in agreement with earlier work on the elementary bodies of influenza virus A. With some preparations a slightly raised base line ahead of the main component in sedimentation pictures suggested a small amount of faster moving material, probably clumped virus particles. When purified preparations were allowed to stand several days at 5° C. a small slower moving boundary appeared as described by Friedewald and Pickels (3). The sedimentation velocity measurements were carried out in a Spinco electrically driven ultracentrifuge equipped with a Philpot-Svensson type of optical

* *We are much indebted to Dr. G. D. Scott of the Physics Department, University of Toronto, for making the electron micrographs.*

PLATE I

FIG. 1. *Refractive index photograph taken after eight minutes at 13,410 r.p.m. in the ultracentrifuge showing sedimenting boundary of influenza virus A in 0.85% sodium chloride solution at pH 7.0. Direction of sedimentation from right to left. Concentration of virus solution 2 mgm. per ml.*

system. A description of the centrifuge has been given by Rhodes and van Rooyen (13).

In eight different preparations of purified virus one ID_{50} for embryonated eggs contained $10^{-14.3}$ to $10^{-15.4}$ gm. of nitrogen.* For five of these preparations the results fell between the limits $10^{-14.85}$ to $10^{-15.10}$ gm. nitrogen per ID_{50}. The influenza virus particle was computed to have a weight of approximately $10^{-15.2}$ gm. assuming a spherical shape of diameter 100 mμ and a density of 1.22 gm. per cc. Since Taylor (14) has found the virus to contain 10.0% nitrogen it was calculated from the above infectivity figures that the purified preparations contained, on the average, about 16 virus particles in one ID_{50}. Friedewald and Pickels (3) determined about 10 particles per ID_{50} in their purified preparations of PR8 virus.

Incorporation of Radioactive Phosphorus into Influenza Virus

In the experiments on the incorporation of radioactive phosphorus into the virus, P^{32}, as inorganic phosphate, was diluted to the desired activity in sterile 0.85% saline adjusted to pH 7 with sodium hydroxide. Unless stated otherwise, 0.2 ml. of the solution of P^{32} was injected into the allantoic cavity of the embryonated egg at an arbitrarily chosen interval three hours following inoculation of influenza virus by the same route. After 48 hr. incubation at 36° C., the allantoic fluid was harvested and the purification of the virus followed the general procedure already given.

Having ascertained that under these conditions the purified virus suspensions contained measurable amounts of P^{32}, it was necessary to ensure that the P^{32} was, in fact, closely associated with the virus and could not be removed by repeated washing of the elementary bodies. The following experiment was designed to settle this point. Total phosphorus and P^{32} estimations were carried out on a number of the fractions obtained during the preparation of the purified radioactive virus and are reported in Table I. The specific activity is defined as the ratio of P^{32} in c.p.m. to total phosphorus in μgm.

Two hundred and nine 11-day embryonated eggs were inoculated with influenza virus. Three hours later each egg received 91,200 c.p.m. of P^{32}, contained in 0.2 ml. of 0.85% saline. During the ensuing 48 hr. incubation period 61 embryos died and were discarded. The remaining eggs were harvested and the allantoic fluid was allowed to stand overnight at 5° C. The agglutinated cells were removed by centrifugation and the supernatant solution (Supernatant A) was discarded. After washing the cells with Ringer – calcium chloride solution, the wash liquid (Supernatant B) was discarded. Fresh Ringer – calcium chloride solution was added and the virus eluted from the cells which were then removed by centrifugation and discarded. This virus solution was centrifuged at 20,000 g for one hour (Supernatant C), and the pellet resuspended in Ringer – calcium chloride and centrifuged at

** These figures were given in error in the preliminary paper as 4×10^{-15} to 5×10^{-14} gm. nitrogen; they should have read 4×10^{-16} to 5×10^{-15} gm. nitrogen.*

TABLE I

SPECIFIC ACTIVITY OF RADIOACTIVE VIRUS DURING PURIFICATION PROCEDURE

Fraction	Volume of fraction, ml.	Total P^{32} c.p.m.	Specific activity of virus, c.p.m./μgm. P
Supernatant A	1010	6,868,000	—
Supernatant B	101	47,000	—
Supernatant C	95	26,000	—
Virus suspension 1	9.5	3850	15.2
Supernatant D	7.6	225	—
Virus suspension 2	7.6	2340	15.0
Supernatant E	5.5	32	—
Virus suspension 3	5.5	1465	16.6
Supernatant F	3.5	Trace	—
Supernatant G	3.5	Trace	—
Supernatant H	3.5	Trace	—
Virus suspension 6	3.5	612	15.9

2000 g for 10 min. to remove large particles and agglutinated virus. The resulting supernatant solution (Virus suspension 1) was subjected to two similar cyles of high and low speed centrifugation to give Supernatants D, E, and Virus suspensions 2, 3, the third virus suspension being made in 0.85% saline. Supernatant F was obtained by centrifuging this suspension at 20,000 g, the virus pellet was resuspended in saline containing 0.01 *M* phosphate buffer, pH 7.0, and allowed to stand 48 hr. at 5° C. to permit any exchange of the P^{32} of the virus with the buffer phosphate. Following centrifugation at 20,000 (Supernatant G) the virus was resuspended in saline, sedimented at high speed (Supernatant H), resuspended in saline, and finally centrifuged for 10 min. at 2000 g to remove the larger particles. The supernatant solution was Virus suspension 6 shown in Table I.

It can be seen from Table I that despite the repeated washings received by the elementary bodies and the opportunity allowed for exchange of P^{32} to occur with phosphate buffer, the specific activities of the various virus suspensions remained essentially constant. This finding indicated that the P^{32} was firmly fixed in the virus. Similar results were obtained in three further such experiments.

Control Experiments on Addition of Radioactive Phosphorus to Infectious Allantoic Fluid

The previous experiment demonstrated that influenza virus grown in the embryonated egg in the presence of P^{32} contained a definite amount of the isotope. There was the possibility, however, that the isotope had not been incorporated into the virus during its actual growth in the cell, but after the virus had been liberated from the cells of the membrane into the allantoic fluid. As described later, a considerable amount of the injected P^{32} remained

in the allantoic fluid, even at the end of the 48 hr. incubation period. Since maximum growth of the virus is almost complete in the first 24 hr. after infection (6), a considerable proportion of the freshly liberated virus would remain in contact with the radioactive allantoic fluid under conditions favorable to exchange of the isotope with the virus phosphorus. To gain information on this point an experiment was carried out in which P^{32} was added to freshly harvested infectious allantoic fluid from which the virus was subsequently isolated and its isotope content determined.

Allantoic fluid was collected from 97 embryonated eggs which had been infected with influenza virus two days previously and allowed to incubate in the usual way. Care was taken to exclude red blood cells when harvesting the fluid. Radioactive phosphate was added to the fluid to give 75,000 c.p.m. per ml., an amount corresponding to that used in the previous experiments on labelling the virus. After standing two days at 5° C. to allow any exchange to take place, chicken red cells were added to make a final 2% suspension. The virus suspension, obtained in the usual way by elution from the cells into saline, was subjected to four cycles of differential centrifugation, the resuspended virus after each step being analyzed for total P and P^{32} as shown in Table II.

TABLE II

SPECIFIC ACTIVITY OF VIRUS AFTER ADDITION OF P^{32} TO INFECTIOUS ALLANTOIC FLUID

Fraction	Volume of fraction, ml.	Total P^{32}, c.p.m.	Specific activity of virus, c.p.m./μgm. P
Infectious allantoic fluid	471	35,200,000	—
Supernatant from agglutinated red cells	460	32,800,000	—
Wash liquid from agglutinated red cells	46	742,000	—
Supernatant 1 after 20,000 g	45.5	120,900	—
Virus suspension 1	13.6	374	2.9
Supernatant 2 after 20,000 g	11.6	276	—
Virus suspension 2	11.6	24	0.3
Supernatant 3 after 20,000 g	9.6	0	—
Virus suspension 3	9.6	48	0.7
Supernatant 4 after 20,000 g	7.6	0	—
Virus suspension 4	7.6	0	—

It is seen that the virus suspension contained a negligible amount of radioactivity as evidenced by its specific activity of 0.3 c.p.m./μgm. P. Such small amounts of radioactivity as were contained in this suspension, where the counting rate was two to three counts above background, were difficult to estimate with any accuracy. It is apparent, however, that little or no exchange had taken place between the virus and inorganic radioactive phosphate. Three further such experiments yielded similar results.

Control Experiments on Addition of Radioactive Phosphorus to Purified Influenza Virus

Although the previous control experiments indicated that influenza virus in infectious allantoic fluid did not take up P^{32} *in vitro* from radioactive phosphate, it was thought of interest to add relatively large amounts of P^{32} to a purified virus suspension to determine the efficiency of the differential centrifugation procedure in removing the isotope.

A suspension of influenza virus in saline was prepared in the usual way with two cycles of differential centrifugation. Radioactive phosphate was added to the purified virus to give a final concentration of 69,600 c.p.m. per ml. of suspension. After standing 24 hr. at 5° C. to permit any exchange, or adsorption of P^{32} on the virus particles, the virus was sedimented at 20,000 g. The supernatant was decanted (Supernatant 1), a small quantity of saline was added to wash down the tube and quickly poured off, and the pellet resuspended in saline. This procedure was repeated three times; total phosphorus and P^{32} estimations were carried out on the various fractions, the results being shown in Table III.

TABLE III

SPECIFIC ACTIVITY OF VIRUS AFTER ADDITION OF P^{32} TO PURIFIED SUSPENSION

Fraction	Volume of fraction, ml.	Total P^{32}, c.p.m.	Specific activity of virus, c.p.m./μgm. P
Virus suspension with added P^{32}	4.6	320,100	—
Supernatant 1 after 20,000 g	4.6	288,500	—
Virus suspension 1	13.8	3160	23.5
Supernatant 2 after 20,000 g	13.8	2900	—
Virus suspension 2	11.8	73	0.74
Supernatant 3 after 20,000 g	11.8	Trace	—
Virus suspension 3	8.5	83	0.89
Virus suspension 4	6.5	51	0.79

From the specific activity figures it is apparent that little of the added P^{32} remained in the second virus suspension. A small amount of radioactivity remained in the virus and was difficult to remove, but too much confidence cannot be placed in the radioactivity estimations for the second, third, and fourth virus suspensions because of the very low counting rate. A further such experiment gave similar results.

Specific Activity of Influenza Virus Grown in Presence of Different Amounts of Radioactive Phosphorus

Fig. 2 shows the variation in specific activity of influenza virus when grown in the embryonated egg in the presence of different amounts of P^{32}. One experiment was carried out to illustrate this point. Two groups of 11-day embryonated eggs were inoculated with influenza virus then with P^{32} after

three hours, 14,850 c.p.m. in each egg in the first group and 64,500 c.p.m. in the second. The remaining data were gathered incidentally from eight other experiments performed during the course of the work. In every case the

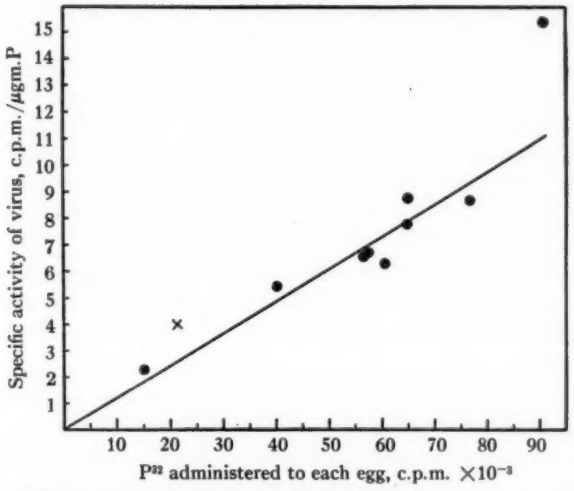

FIG. 2. *Relationship of specific activity of purified radioactive virus to amount of radioactive phosphorus administered.*

specific activity of the virus was determined after purification in the usual way with two cycles of differential centrifugation, except for one preparation which received only one cycle of centrifugation. The latter preparation is represented by a cross in the figure.

It is seen that the specific activity of the virus rises in a linear manner with the amount of injected P^{32} up to at least 91,200 c.p.m. in each egg.

Effect of β-Radiation from Radioactive Phosphorus on the Embryo and on the Growth and Properties of Influenza Virus

During the course of the work it was found that the death rate of embryonated eggs injected with influenza virus A by the allantoic route might be as high as 6% during the succeeding 48 hr. incubation period and on the average was about 3-4%. When radioactive phosphorus was injected also, by the allantoic route, the embryo death rate increased until, with 70,000 c.p.m. injected into each egg, 10 to 30% of the embryos died; there was a large variation in the number of embryo deaths from one experiment to another. It seems probable that many of these deaths resulted from the effect of β-radiation, the dead embryos often appearing markedly haemorrhagic. This finding put a practical upper limit on the amount of isotope which could be injected into each egg and, consequently, an upper limit on the amount of isotope which could be incorporated into the influenza virus.

In spite of the fact that, in several experiments, many of the embryos died from radiation injury, the yield of influenza virus from the surviving eggs was no less than from eggs which had not received P^{32}. This was confirmed on several occasions by doing infectivity titrations and CCA tests on the pooled allantoic fluid from eggs which had survived the injection of up to 150,000 c.p.m. of P^{32}.

In addition, the properties of purified influenza virus containing radioactive phosphorus, up to a specific activity of 15 c.p.m./μgm. P did not seem to be measurably different from those of normal virus. The appearance in electron micrographs and the sedimentation constant were unchanged. Infectivity figures given in an earlier section for eight different preparations of purified virus include three radioactive preparations; there was no significant difference between the radioactive and normal virus preparations.

It would appear, therefore, that relatively severe irradiation of the allantoic membrane, where influenza virus multiplication is presumed to occur, did not interfere appreciably with the virus growth, nor did it alter the properties of the purified virus.

Specific Activity of Influenza Virus with Varying Intervals Between Virus and Radioactive Phosphorus Inoculation into Embryonated Eggs

Previous experience had indicated that the maximum rate of growth of the PR8 strain of influenza virus A occurred during 24 hr. after inoculation of the egg. It was assumed that if P^{32} were injected into the allantoic cavity while the virus was rapidly growing, conditions should be favorable for incorporation of the isotope into the virus, since the cells of the allantoic membrane should be in intimate contact with P^{32} during this period. Consequently, in all experiments involving labelling of influenza virus with P^{32} so far reported in this paper, radioactive phosphorus was injected into embryonated eggs three hours following inoculation of virus.

There was a possibility, however, that P^{32} uptake by the growing virus might be much greater if some of the normal phosphorus containing constituents in the membrane cells were labelled through injection of the isotope at some time prior to infection with influenza virus. Therefore, a single experiment was carried out in which a group of 50 embryonated eggs was inoculated with P^{32} at each of the intervals, 48 hr. before, 24 hr. before, a few minutes after, and 24 hr. after infection with 10-100 ID_{50} of influenza virus. Each egg of the four groups received 76,000 c.p.m. of the isotope. After incubation of the eggs the virus from each group was purified, as usual, analyzed for P^{32} and total phosphorus, and the specific activity was calculated. The results are shown in Table IV.

It would appear that when P^{32} was injected into eggs 48 hr. previous to virus infection, the specific activity of the virus was about 22% higher than when the isotope was administered almost simultaneously with the virus. When the isotope was injected 24 hr. after infection the specific activity of the virus was very low, and it is worth noting that this finding supports

strongly the previous indication that little or no exchange occurs between virus and P^{32}. In this case the virus, freshly liberated from the allantoic membrane during the 24 hr. following infection, remained in contact, *in vivo*,

TABLE IV

SPECIFIC ACTIVITY OF INFLUENZA VIRUS WHEN P^{32} INJECTED INTO EMBRYONATED EGGS AT DIFFERENT TIMES WITH RESPECT TO TIME OF VIRUS INOCULATION

Time of injection of P^{32}	Specific activity of purified virus c.p.m./μgm. P
48 hr. before virus	10.5
24 hr. before virus	9.8
Same time as virus	8.6
24 hr. after virus	0.56

with allantoic fluid containing large amounts of P^{32} for a further 24 hr. The low specific activity of the virus from this group of eggs, compared to the very much higher specific activities obtained in the other three groups, indicates that the virus is labelled with the isotope only during its growth in the cells of the membranes. Even the low specific activity observed in the fourth group could be accounted for by the relatively small amount of virus multiplication which occurred during the second 24 hr. period following infection of the eggs.

Disappearance of Radioactive Phosphorus from Allantoic Fluid and from Yolk Sac of Embryonated Egg

During the course of the work it was felt necessary to obtain information on the amount of P^{32} remaining in the allantoic fluid at various intervals following injection of P^{32}. Experiments designed for this purpose were carried out as follows:

Each of a number of 11-day embryonated eggs was injected with 0.2 ml. of P^{32} solution by the allantoic route, the isotope being dissolved either in saline or in 0.1 *M* phosphate buffer, pH 7. Within 5 to 10 min. after injection the allantoic fluid of five eggs was harvested and the total volume of fluid measured. Radioactivity estimations were carried out on the combined fluids. Control experiments, carried out by diluting a saline solution of known P^{32} content in normal allantoic fluid and measuring the activities of various dilutions, indicated that the presence of allantoic fluid did not interfere with the method of assay. The remaining eggs were incubated at 36° C. and, at intervals, groups of five eggs each were treated in the same manner as above.

In all, 10 such experiments were performed, the amount of P^{32} injected per egg varying between 8700 c.p.m. to 40,900 c.p.m. from one experiment to

another. The results of two of the experiments, which are representative of the remainder, are shown in Fig. 3.

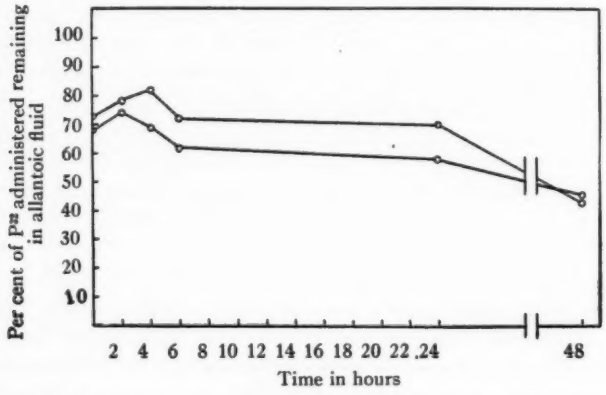

FIG. 3. *Rate of disappearance of P^{32} from the allantoic fluid of 11-day embryonated eggs.*

In none of the experiments was more than 90% of the administered P^{32} recovered from the groups of eggs harvested at zero time. This was probably partly due to the fact that it was impossible to harvest the allantoic fluid quantitatively. The slight increase in P^{32} recovered in the allantoic fluid, shown between two to four hours in Fig. 3, was observed in each of the remaining eight experiments, although in some cases the increase was not seen until four to six hours after injection of isotope. These findings were not considered of immediate importance and were not investigated further. In two experiments, 42% and 51% of the administered isotope was recovered after 72 hr.

Although similar experiments were not done with influenza virus infected eggs, it was observed incidentally during another part of the work that 40% to 50% of the administered P^{32} was recovered after 48 hr. from the allantoic fluid of infected eggs.

Two experiments were carried out to determine whether P^{32} injected into the yolk sac of 11-day embryonated eggs would appear rapidly in the allantoic fluid. Since the technique of inoculation into the yolk sac is not easy it was necessary to ensure that the isotope was placed in the desired position. Consequently the P^{32} solution, 46,000 c.p.m. into each egg, was made up in 2% trypan red solution for inoculation. It had been determined previously that trypan red remained indefinitely in the yolk sac and that it was innocuous to the developing embryo. On harvesting the eggs the allantoic fluid was used only from those eggs in which the dye was localized in the yolk sac.

After 72 hr., only 1.5% of the administered P^{32} had appeared in the allantoic fluid.

Discussion

It is considered that the suspensions of purified virus handled throughout this work consisted almost entirely of the elementary bodies of influenza virus. Sedimentation (3, 15), electron microscope (15, 17), infectivity results (3), and chemical analysis (5, 14) were in accord with the observations of previous workers and together suggested that little or no soluble or particulate matter other than virus was present in these preparations.

It would appear that the P^{32} in the radioactive virus was, in fact, incorporated into the structure of the virus. This is indicated by the findings that virus does not exchange with P^{32} and that the isotope in labelled virus cannot be removed by repeated washings of the elementary bodies. Further evidence was offered by the observation, made during chemical analysis of radioactive virus, that both the phospholipid and nucleic acid fractions of purified virus contained P^{32} (5).

In radioactive tracer experiments with biological materials, it is essential that the radiation from the isotope should not interfere with the metabolism of the system under observation. In such studies, it is generally easy to exclude radiation effects since only minute amounts of tracer are required. However, for the purpose of further study of the labelled influenza virus, it was necessary to obtain virus with high specific radioactivity. It became apparent early in the work that in order to achieve this end, relatively large amounts of P^{32} would have to be injected into the virus infected egg. In many experiments, when 450-600 μrd. of P^{32} or more were injected into each egg, a significant proportion of the embryos died.

It was considered that these deaths may have resulted from some toxic material present in the P^{32}. However, the isotope solutions, as received from the National Research Council, were usually diluted at least one thousandfold before administration to the eggs in 0.2 ml. amounts. It was thought improbable that any compound would be present in sufficiently high concentration in the original solution to cause the high mortality observed in some of the groups of fertile eggs. It was therefore presumed that death of the embryos was in large measure due to β-radiation injury.

In spite of these observations it is noteworthy that in the infected eggs which survived the injection of as much as 1300 μrd. of P^{32} the yield of virus was not appreciably less than from eggs which had not received the isotope. It was noted also that the specific activity of purified radioactive virus rose linearly with increasing amounts of P^{32} administered to the infected eggs up to at least 775 μrd. for each egg. If radiation were interfering with the growth of virus both the yield and specific activity of the virus might be expected to decrease rapidly with increasing amounts of isotope administered. It would thus appear that the mechanisms involved in the growth of influenza virus are relatively resistant to β-radiation.

One of the main points we had hoped to investigate with the radioactive virus was the fate of the isotope when the virus was growing in the allantoic

membrane. So far such a study has been precluded by the small amount of P^{32} which has been introduced into the virus. It can be calculated that the specific activity of the radioactive virus would have to be increased at least a thousandfold over the highest value yet obtained in order to study this problem to any advantage. While there would seem to be little hope of obtaining influenza virus with such a high specific activity by the methods reported here, it is a subject for investigation whether the activity might be increased substantially by administering the isotope by some route other than by the allantoic sac.

The infectivity, sedimentation characteristics, and morphology of the purified radioactive virus were not significantly different from those of unlabelled virus. It was calculated that, for influenza virus having a specific activity of 1.29 μrd. per μgm. P (15 c.p.m. per μgm. P), the proportion of radioactive phosphorus atoms to total phosphorus atoms in the virus was 1 to 8.4×10^9. That is, on the average, one radioactive phosphorus atom was present for every 66,500 virus particles. Therefore, even if the properties of labelled virus were markedly different from those of unlabelled virus, the presence of such a small proportion of radioactive virus particles would hardly be expected to cause a measurable change in the biological or physical properties of the virus preparation. Thus it is not possible to state as yet whether labelled virus behaves in the same manner as unmarked virus.

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